AN EVALUATION OF IMMUNOHISTOCHEMISTRY FOR STUDYING CYCLIC NUCLEOTIDES IN THE CENTRAL NERVOUS SYSTEM, WITH PARTICULAR REFERENCE TO GUANOSINE 3',5'-MONOPHOSPHATE

BY

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DEDICATION

To my wife Dawn, forever

ABSTRACT

An immunofluorescent technique has been developed with the aim of histochemically localizing cyclic AMP and cyclic GMP in the C.N.S., and determining the cellular sites where biochemical changes in tissue levels occur.

Initial experiments revealed that the immunoglobulin fraction of non-immune sera bound non-specifically to C.N.S. tissue, via a weak charge attachment between IgG and basic tissue proteins.

Specific antibodies were raised in rabbits by immunization with succinyl cyclic nucleotide-protein conjugates, and radioimmunological techniques with [^{3}H] tracers were used to study binding characteristics. Of the large number of cyclic GMP antibodies studied in detail, specific staining of astrocytic fibres and capillaries (a contrasting localization to that of cyclic AMP), was found with only a small number of antibodies, although these could not be identified on the basis of titre, avidity or specificity however.

Differences between RIA and immunohistochemistry, and different staining patterns with individual cyclic GMP antibodies, have been discussed as resulting from stereochemical differences between free and tissue-bound nucleotide. This may also explain why cyclic nucleotide antibodies have satisfied the criteria of specificity for RIA, but not for immunofluorescence.

'In vivo' and 'in vitro' biochemical techniques, coupled with cyclic GMP immunofluorescence, failed to localize semi-quantitative changes in intensity and/or distribution of staining, under conditions where total nucleotide levels were significantly altered.

Quantifying cyclic nucleotide losses from frozen tissue sections, it was determined that more than 80% of cyclic GMP was lost during buffer-washing, and that the tissue-bound pool was unchanged when total levels were elevated - a possible explanation for the inability to localize biochemical changes using immunofluorescence.

Recently developed antibodies to cyclic GMP-dependent protein kinase, and other cyclic nucleotide receptor proteins, were used to investigate the binding, and determine the function of, the pool of cyclic nucleotides localized in C.N.S. tissue sections by immunofluorescence.

Statement in terms of regulation 2.4.15 of the Postgraduate Study Programme of the University of Edinburgh.

I declare that this thesis was totally composed by myself, and that all the experimental work described herein was performed by myself, with the following exceptions:

a) Cyclic GMP determinations in frozen tissue sections (chapter V) were performed by Miss S Dickison.

b) Immunofluorescent localization of cyclic GMP, cyclic AMP and cyclic GMP kinase in areas of the C.N.S. excluding the cerebellum and spinal cord were carried out together with Dr I Laszlo.

R.D.F. CUMMING

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NOTES AND ABBREVIATIONS

Cyclic AMP	-	adenosine 3',5'-monophosphate
Cyclic GMP	-	guanosine 3',5'-monophosphate
ATP	-	adenosine triphosphate
C.N.S.	-	central nervous system
CDR	-	calcium-dependent regulator protein
PBS	-	phosphate-buffered saline
RIA	-	radioimmunoassay

All other abbreviations used in this thesis, are those in common scientific usage.

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CHAPTER I

INTRODUCTION

CHAPTER I

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1. CYCLIC AMP AND ITS ROLE IN THE CENTRAL NERVOUS SYSTEM - LITERATURE REVIEW.

In 1957 Rall et al discovered a low molecular weight heat stable 'factor' which accumulated in particulate fractions of liver homogenates exposed to the hormone adrenaline (1). Since this 'factor' alone could stimulate glycogen breakdown in the soluble fraction of homogenates, Rall et al proposed that this 'factor' mediated hormonal stimulation of glycogenolysis. Subsequent experiments by Lipkin et al (2) identified this 'factor' as adenosine 3',5'-cyclic monophosphate (cyclic AMP), a nucleotide related to ATP, the ubiquitous energy source of the cell. Sutherland et al (3) soon found that the membranes of liver cells, and many other cells, contained an enzyme which they named adenylate cyclase, capable of converting ATP to cyclic AMP. Since ATP is found almost entirely in the cytoplasm it was reasoned that at least part of the enzyme must face the interior of the cell to synthesize cyclic AMP.

Exposure of liver cells to adrenaline was found to substantially increase the rate of conversion of ATP to cyclic AMP. This observation, subsequently found with a large number of peptide hormones in different tissues, indicated that there was a functional link between the binding of these hormones at specific receptor sites on the outside of the membrane, and the activation of adenylate cyclase to elevate cyclic AMP concentration on the inside (4). This mechanism, transducing extracellular signals to the intracellular 'milieu', is fundamentally different from the actions of the other class of hormones, steroids, which are able to

diffuse through the cell membrane, carrying their 'message'. It is now generally accepted that cyclic AMP acts as a 'second messenger' molecule relaying the message of the hormone (1st message) from the cell membrane to the internal 'biochemical machinery' of the cell in order to trigger the physiological response. A variety of biochemical steps are involved in this information transfer which allows amplification of the initial hormonal signal at each step (5).

Two early observations suggested that cyclic AMP might mediate the physiological actions of neurotransmitters in the nervous system in a way analagous to hormonal effects. Adenylate cyclase was found to have its highest activity in the C.N.S (3), and the putative neurotransmitter noradrenaline, stimulated cyclic AMP production in homogenates of brain tissue (6). The following review considers the very large amount of information on cyclic AMP in the nervous system. Whilst most interest has focused on the role of cyclic AMP in mediating post-synaptic potential changes induced by neurotransmitters involved in propogation of the nerve impulse, other possibilities exist and post-synaptic events of longer time-course e.g. regulation of protein synthesis, will be considered. Discussion on possible non-synaptic roles of this nucleotide will also be included.

1.1. The enzymatic machinery involved in the synthesis, action and degradation of cyclic AMP in the C.N.S.

1.1.A. Adenylate cyclase

Adenylate cyclase is the membrane bound enzyme catalysing the formation of cyclic AMP from the substrate ATP, a process which

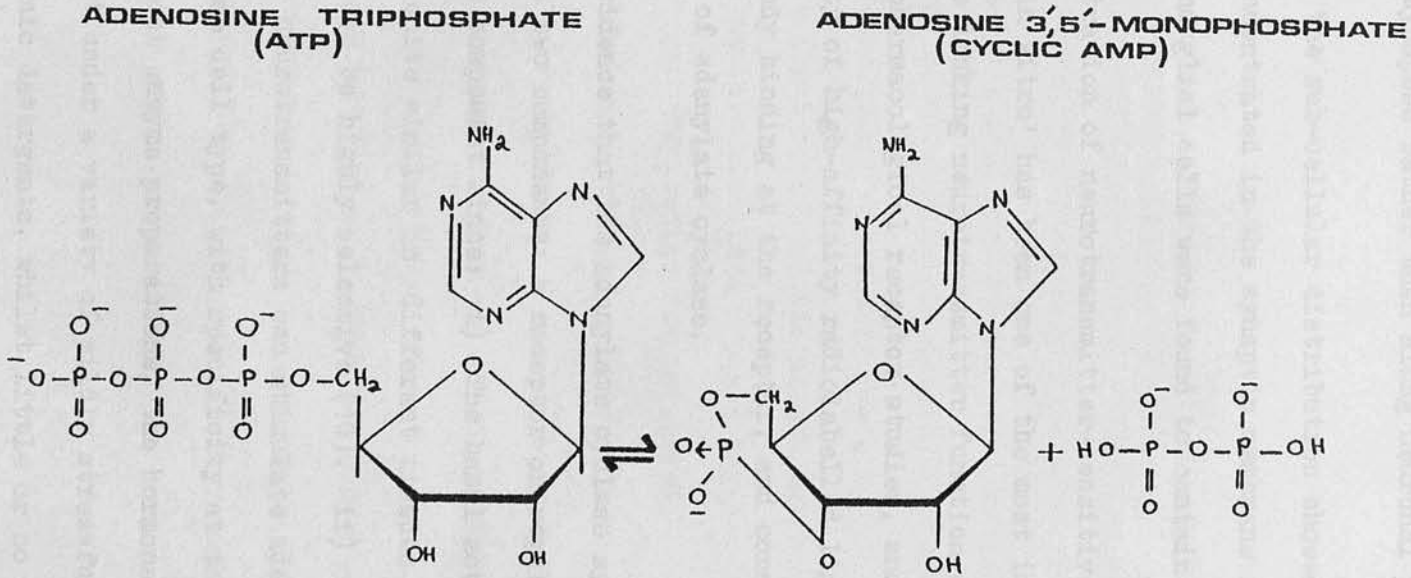


Figure 1 Synthesis of cyclic AMP from ATP

is dependent upon Mg^{2+} (3). The C.N.S. contains very high levels of this enzyme with considerable regional and species variation in concentration. In rat the overall activity is highest in cerebellum followed by cortex. Activity is higher in grey matter than white, suggesting localization in cell bodies or synapses rather than along neuronal processes (7).

Studies on the sub-cellular distribution showed the enzyme to be most concentrated in the synaptic membrane fraction (8). Both neuronal and glial cells were found to contain the enzyme (9).

The demonstration of neurotransmitter-sensitive adenylate cyclases 'in vitro' has been one of the most important pieces of evidence linking neurotransmitter function with cyclic AMP. Extensive pharmacological receptor studies, and the recent availability of high-affinity radiolabelled ligands have been used to study binding at the receptor, and consequential activation of adenylate cyclase.

There is evidence that the adenylate cyclase system is composed of at least two components: a receptor or regulatory unit, and a catalytic component since: i) The basal activities of the enzyme are quite similar in different tissues whilst hormonal regulation can be highly selective (10). ii) Two or more hormones or neurotransmitters can stimulate adenylate cyclase from the same cell type, with specificity at the receptor (10). iii) For most enzyme preparations, the hormonal sensitivity is readily lost under a variety of mildly stressful conditions, e.g. non-ionic detergents, whilst little or no change in basal activity may occur (3).

A number of models have been proposed for the arrangement of the receptor and catalytic units of adenylate cyclase in the membrane e.g. (11, 12). The key question is whether the units are on the same, or different molecules. Recent evidence from cell lines expressing the different units in different ways, suggests that they are distinct (13).

Cell hybridization in turkey erythrocytes with one cell line expressing a β-catecholamine receptor and inactive adenylate cyclase, and another expressing an active adenylate cyclase but no β receptor, showed a β receptor-sensitive adenylate cyclase. These 'fusion' results and other studies with glioma and neuroblastoma cells (14) support the 'mobile receptor hypothesis' which envisages hormone-receptor complexes separated from adenylate cyclase molecules, which can combine as a result of their location in the dynamic lipid matrix of the plasma membrane. Other agents are able to stimulate adenylate cyclase besides hormones and neurotransmitters. Millimolar concentrations of fluoride ions are able to stimulate adenylate cyclase in broken cell preparations but not whole cells (although they almost certainly enter cells); the activation is markedly temperature-dependent (15) and appears to modify the requirement of the enzyme for Mg^{2+} (16). Presumably fluoride ion activation 'by-passes' the regulatory unit of the enzyme and causes direct activation of the cyclase. Cholera toxin is another compound which probably acts like fluoride, causing irreversible activation of the adenylate cyclase (17).

Recent work on brain, has shown that a snake venom (Russel viper)

can uncouple the dopamine receptor from striatal adenylate cyclase (18). The venom which irreversibly blocks the receptor is also able to eliminate the response of adrenal adenylate cyclase to stimulation by ACTH. Dopamine sensitivity can be restored to venom-treated caudate homogenates by treating with guanine nucleotides. GTP and other guanine nucleotides have been found to stimulate the responsiveness of adenylate cyclase to dopamine (19) and other hormones and neurotransmitters, perhaps due to allosteric regulation of the enzyme.

Whilst the fluoride ion activation may be primarily of theoretical interest, it may be of more relevance physiologically that micromolar concentrations of calcium ions can inhibit brain adenylate cyclase while millimolar concentrations stimulate the basal but not the hormonal stimulated cyclase (20). Calcium ions can also modify the degradative enzyme for cyclic AMP, phosphodiesterase (see section 1.1.C.), by binding to a specific calcium-binding protein. There is the intriguing possibility that this protein might serve to regulate both the synthesis and degradation of cyclic AMP. Basal and hormonal stimulated adenylate cyclase require millimolar concentrations of ionic co-factors, usually satisfied by Mg^{2+} but Mn or Co may substitute (21). Low concentrations of other metals e.g. Pb, Hg, Cu, La are potent inhibitors (22, 23, 24). The effects of Pb, Hg and Cu which bind to cysteine residues, are reversed by sulphydryl reducing agents. The importance of phospholipids as factors affecting adenylate cyclase is born out by the observation that hormonal sensitivity can be restored to adenylate cyclase inactivated with detergent treatment, by addition of specific phospholipids in a number of tissues (25).

Studies on the developing C.N.S. show that the activity of adenylate cyclase increases during post-natal life and that the capacity for hormonal stimulation is demonstrable after a basal level of adenylate cyclase has been established (26). The development of different neurotransmitter-sensitive adenylate cyclases may vary e.g. the serotonin-sensitive enzyme has maximal activity at birth, decreasing to negligible levels at six weeks (27), strongly suggesting a role in development.

1.1.B. Cyclic AMP-dependent protein kinases and phosphorylation

The physiological effects of cyclic AMP in mammalian systems are now all believed to be mediated by binding to specific protein kinases, and catalysing the phosphorylation of specific proteins (5).

In brain, cyclic AMP kinases are found in both soluble and particulate fractions; the particulate cyclic AMP kinases are associated with nerve ending membrane fractions which can serve as substrates for the phosphorylation reactions (28). It should be noted at this point that cyclic AMP-independent kinases are also found in brain (29). Cyclic AMP activates the cyclic AMP kinase by binding to an inhibitory sub-unit which then causes dissociation of an active catalytic unit. Mammalian kinases are thought to exist in at least two forms with different inhibitory units, known as RI and RII but a common active catalytic unit. Membrane bound cyclic AMP kinase in brain might be type II since evidence from other tissues suggests that the type II form binds tightly to membrane components (30, 31). Greengard et al (32) have shown that in membrane fractions from the C.N.S. binding of cyclic AMP to the kinase specifically phosphorylates two proteins in the synaptic membrane. These proteins, named protein I and protein II have molecular weights

of approximately 86,000 and 49,000 respectively. Whilst protein II is found in membrane fractions from a number of tissues, protein I is found only in membrane fractions containing synapses. Protein I is absent from the brains of fetal rats at stages of development before synapse formation occurs between nerve cells, appearing when synaptic complexes form. Furthermore, recent evidence using the neurotoxin kainic acid, has been found to drastically reduce levels of protein I in rat striatum (33). The phosphorylation and dephosphorylation of this protein are extremely rapid, being an ideal mechanism to change post-synaptic membrane potentials. In incubated slices of rat brain, protein I is found almost entirely in the de-phosphorylated form and recently it has been shown that certain drugs and depolarizing agents can change the phosphorylation state of this protein (34). Dephosphorylation occurs via phosphoprotein phosphatases, also found in the synaptosomal membrane.

Having commented upon the rapid phosphorylation/dephosphorylation mechanism it should be noted that very fast transmission at certain synapses e.g. neuromuscular junction, is not mediated via cyclic AMP. This is not surprising since, as already described, a number of steps are involved between neurotransmitter interaction and phosphorylation, which would take a long time course by synaptic transmission standards.

Protein phosphorylation is presumed, but has not been directly shown, to cause changes in ionic permeability generating post-synaptic potential changes in the C.N.S. The problems are technical, due to the short time courses involved, definitive evidence probably being obtained only when the phosphorylating machinery can be 'dissected out' and reimplanted into an artificial membrane for study. However, indirect evidence has come from non-neural tissues. In the avian erythrocyte for example (35) catecholamine-induced phosphorylation of a/

single membrane protein, mediated by cyclic AMP, agrees well both temporaly and on dose-dependency, with the increase in sodium ion transport across the membrane. Furthermore, agents that block this increase in sodium ion transport, also block catecholamine-induced phosphorylation.

The presence of cyclic AMP kinase in the soluble fraction of the cell and also in the nucleus, suggests that cyclic AMP might control post-synaptic events with a longer time course than for permeability changes. Translocation of the kinase sub-units from cytoplasm to nucleus has been suggested in a number of tissues e.g. ovary, adrenal medulla and liver (36). In the nucleus, histone phosphorylation via cyclic AMP-dependent kinase can modify histone binding to D.N.A. and hence affect gene expression. A system is therefore available for control of protein synthesis by translocation of cyclic AMP kinase (37).

Two heat-stable inhibitors of cyclic AMP protein kinase have been described (38), one of which binds to the catalytic unit of the kinase, whilst the other antagonizes the interaction between kinase and the phosphate-acceptor protein.

1.1.C. Cyclic AMP phosphodiesterase.

The actions of cyclic AMP are terminated not only by dephosphorylation of specific proteins, but also by removal of free cyclic AMP. Cyclic AMP is degraded by the enzyme phosphodiesterase, which cleaves the 3'5' phosphodiester bond to form 5' AMP. It has been found that when cyclic AMP is complexed to the kinase, and therefore in its physiologically active form, it cannot be degraded by the enzyme (39).

Brain contains considerable phosphodiesterase activity and regional distribution shows high levels in cortex, striatum and limbic forebrain, with much lower levels in cerebellum and brain stem (40). Subcellular distribution shows high concentrations in synaptic membranes, synaptic vesicles and microsomes (8, 41, 42).

Phosphodiesterase is not a single enzyme but a class of enzymes (isoenzymes) that have different physical and chemical properties. The multiple forms are found in different amounts in various areas of mammalian brain. All of the isoenzymes will hydrolyse cyclic AMP and its related nucleotide cyclic GMP (see section 2.2.) if the nucleotides are present in high enough concentration. However, at physiological concentrations, certain of the enzymes show specificity for one or other nucleotide, whilst at least one type hydrolyses both nucleotides equally well (43).

Phosphodiesterase is found with a soluble and particulate distribution, most work having been carried out on the soluble form. The activity of at least one isoenzyme of phosphodiesterase is regulated by the ubiquitous calcium binding protein, CDR (44). In the presence of calcium, CDR complexes with phosphodiesterase increasing enzyme velocity and/or decreasing the Km of the enzyme for substrate.

Whilst calcium-dependent activation increases the ability of phosphodiesterase to hydrolyse both cyclic AMP and cyclic GMP, the effect on cyclic GMP hydrolysis may be of more importance physiologically (see section 2.2.)

Regulation of soluble phosphodiesterase has been shown recently by Uzonov et al (45),who demonstrated that cyclic AMP-dependent protein phosphorylation caused the release of particulate CDR into the cytosol

to stimulate hydrolysis of cyclic AMP; the release was calcium-independent and could not be triggered by cyclic GMP-dependent phosphorylation.

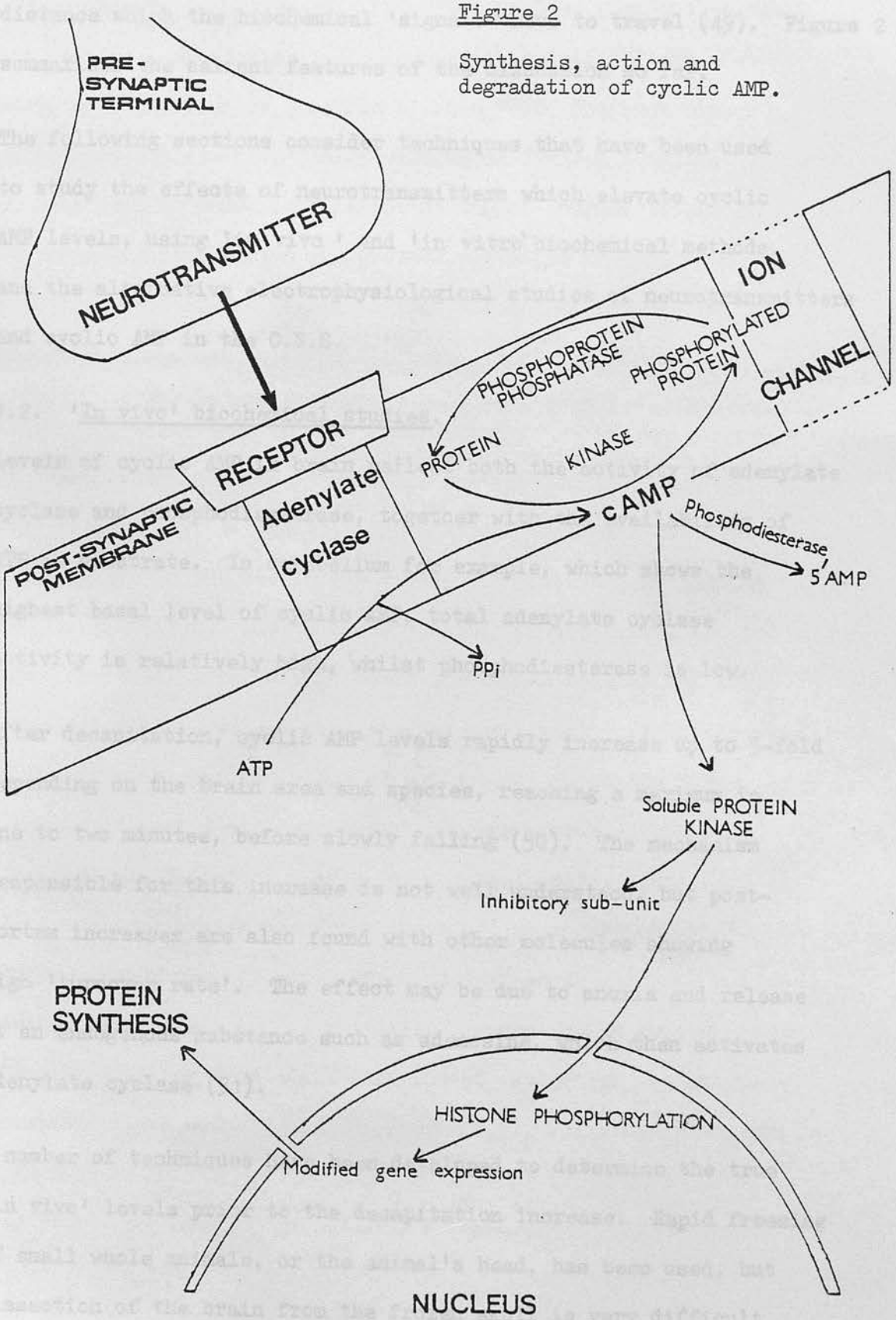
A variety of compounds e.g. methyl xanthines, papaverine, puromycin, inhibit phosphodiesterase, many of which show behavioural effects. The inhibitors are important since they prolong the action of cyclic AMP by preventing its degradation, and should therefore prolong the physiological effects of neurotransmitters, if the nucleotide is acting as a 'second messenger'. Problems arise however from inhibitors differentially inhibiting certain of the phosphodiesterase isoenzymes (46).

Whilst phosphodiesterase is the only proven mechanism for degrading cyclic AMP, a number of observations suggest that the levels of cyclic AMP may be reduced by active or passive release from cells, since cyclic AMP is transported into cerebrospinal fluid (C.S.F.) by a probenecid-sensitive mechanism. Probenecid is a competitive antagonist of organic acid transport which suggests that the efflux is a specific process (47). The C.S.F. concentration of cyclic AMP is increased following intraventricular injections of adrenergic agents. An uptake mechanism may also control levels since cortical slices have been shown to have a high affinity, temperature-sensitive uptake mechanism (48).

The preceding discussion has considered the mechanism by which cyclic AMP is synthesized, acts and is broken down. Perhaps the most important point is the compartmentalization of the system for post-synaptic events, since every component of the 'machinery' is found in the synaptosomal membrane.

Figure 2

Synthesis, action and degradation of cyclic AMP.



Perhaps the components exist in macromolecular complexes to limit still further, and therefore make more efficient, the distance which the biochemical 'signals' have to travel (49). Figure 2 summarizes the salient features of the discussion so far.

The following sections consider techniques that have been used to study the effects of neurotransmitters which elevate cyclic AMP levels, using 'in vivo ' and 'in vitro' biochemical methods, and the alternative electrophysiological studies of neurotransmitters and cyclic AMP in the C.N.S.

1.2. 'In vivo' biochemical studies.

Levels of cyclic AMP in brain reflect both the activity of adenylate cyclase and phosphodiesterase, together with the availability of ATP as substrate. In cerebellum for example, which shows the highest basal level of cyclic AMP, total adenylate cyclase activity is relatively high, whilst phosphodiesterase is low.

After decapitation, cyclic AMP levels rapidly increase up to 5-fold depending on the brain area and species, reaching a maximum in one to two minutes, before slowly falling (50). The mechanism responsible for this increase is not well understood, but post-mortem increases are also found with other molecules showing high 'turnover rate'. The effect may be due to anoxia and release of an endogenous substance such as adenosine, which then activates adenylate cyclase (51).

A number of techniques have been developed to determine the true 'in vivo' levels prior to the decapitation increase. Rapid freezing of small whole animals, or the animal's head, has been used, but dissection of the brain from the frozen skull is very difficult

after this, and more importantly deep brain areas may freeze more slowly than superficial areas, yielding inaccurate results. 'Freeze-blowing' (52) freezes the brain in less than one second, but requires exposure of the organ before sacrifice, and also prevents dissection of specific brain areas. The most common method now employs high energy microwaves focused onto the animals head, to cause rapid inactivation of enzymes within a few seconds, prior to the post-mortem elevation of cyclic AMP (53). A disadvantage of the method is that the animal may need to be restrained in order to focus the beam, which may stress the animal, and affect cyclic nucleotide levels (145).

Employing techniques to prevent the post-mortem increase, a very large number of agents and conditions have been found to increase cyclic AMP levels 'in vivo', with marked regional and species variation. Due to the complexity of the C.N.S. and the large number of effects that these treatments have on different neurotransmitter systems, such studies are probably of little use in determining specific roles of the nucleotide in the C.N.S. A number of agents and conditions altering cyclic AMP levels 'in vivo' are therefore listed for interest only: adenosine, noradrenaline, histamine, prostaglandins, steroids, morphine, phosphodiesterases, ethanol, electric foot-shock stress. For further details see (55). A similar argument holds for behavioural effects resulting from administration of cyclic AMP, or its more permeable lipid-soluble analogue, dibutyryl cyclic AMP, into the C.N.S.

1.3. 'In vitro' biochemical studies.

Broken-cell homogenates of brain tissue have been extensively used to study neurotransmitter-sensitive adenylate cyclases. In general, the degree of responsiveness of the cyclase is less in nerve tissue homogenates than in other tissues, observations which hampered early work. Typically, homogenates of C.N.S. tissues show, at most, a 2-4 fold increase in adenylate cyclase activity when exposed to putative neurotransmitters. The low responsiveness may be due to calcium ions released from intracellular sites on homogenization (54) which activates basal, but not stimulated, cyclases. This would cause the measured activation to be less than actually occurred. Additionally, the basal adenylate cyclase measured in homogenates, represents the sum total of different neurotransmitter-sensitive adenylate cyclases. Stimulation of one type might be 'diluted out' by non-responding pools (10).

A number of putative neurotransmitters have been shown to be effective in stimulating adenylate cyclase in homogenates of C.N.S. tissue e.g. dopamine, noradrenaline, serotonin, substance P, prostaglandins, histamine (10). A marked degree of variation in response is found between species and brain area. Good correlation is generally found between magnitude of response of neurotransmitter-sensitive cyclase and extent of innervation by that neurotransmitter of the particular brain area, used for homogenates. The dopamine-sensitive adenylate cyclase will be discussed in some detail to illustrate this, and to demonstrate how pharmacological studies have been used to study the relationship between this neurotransmitter receptor, and adenylate cyclase.

Dopamine-sensitive adenylate cyclases have been found in brain areas such as striatum rich in dopamine nerve terminals(as visualized by 'Falk-Hillarp' fluorescence histochemistry),and rich in dopamine receptors as measured using high affinity radiolabelled ligands. The cyclase is stimulated by low concentrations of dopamine and structurally related analogues (56), and has its highest concentrations in synaptic membrane fractions (21).

In general, there is good correlation between the potencies of anti-psychotic drugs to inhibit dopamine-sensitive adenylate cyclase in limbic forebrain homogenates and their clinical potencies (57). There are exceptions to this correlation however, e.g. haloperidol is less effective in inhibiting dopamine-sensitive adenylate cyclase than chlorpromazine, but haloperidol is much more effective clinically (58). Whilst considerations such as differences in solubility and absorption might explain this difference, haloperidol and related compounds have been reported to be potent inhibitors of the stimulation-evoked release of dopamine from nerve endings (58). This pre-synaptic action, by decreasing the amount of dopamine available to the post-synaptic receptors would tend to augment the effect of these drugs on blocking dopamine-sensitive adenylate cyclase, thus leading to enhanced clinical potency.

A biochemical lesion in the dopaminergic pathway leading from the substantia nigra to the corpus striatum has been found to be responsible for the movement disorder known as Parkinsonism. Degeneration of the dopaminergic nerve terminals in striatum reduces the amount of dopamine available to interact with its

receptors and the receptors become 'supersensitive'. The symptoms of this disorder are tremor, rigidity and delay in initiation of movement (59). High doses of anti-psychotic drugs whose prime effects lie in blocking dopamine receptors in limbic forebrain, sometimes result in Parkinson-like side effects presumably since they also block striatal dopamine receptors (60). In general there is good correlation between the degree of these 'extra-pyramidal' side effects and inhibition of dopamine-sensitive adenylate cyclase in broken-cell striatal homogenates. The exceptions may be due to actions of anti-psychotics in blocking muscarinic receptors in the C.N.S. (61).

The increased sensitivity of dopamine-sensitive adenylate cyclase to dopamine and its agonists which is found experimentally after chemical lesions of the nigro-striatal pathway (62) with 6-hydroxydopamine demonstrates a very important function of cyclic AMP. Not only is cyclic AMP responding post-synaptically to neurotransmitter, but it is also being modified by the sensitivity of the receptor.

The use of dopamine receptor agonists and antagonists have shown that there is a good correlation between receptor occupation, and activation or inhibition of adenylate cyclase. This correlation has led to a new methodology for the rapid screening and development of drugs as dopamine receptor agonists (for replacing the dopamine deficiency in Parkinsonism), and antagonists (for use as anti-psychotics).

The homogenate, whilst obviously of use as demonstrated above, is limited by its degree of responsiveness. The respiring incubated

brain slice has been used extensively for studying the actions of neurotransmitters on cyclic AMP and evokes larger increases in cyclic AMP levels. Since the cells are intact and respiring, the slice is closer to the 'in vivo' situation than the broken-cell homogenate, and allows fine control to be made over the external 'environment'. Its disadvantage with respect to the homogenate, is that neurotransmitters causing elevations in tissue levels of cyclic AMP cannot automatically be attributed to direct activation of adenylate cyclase, even if a phosphodiesterase inhibitor is present. Increased levels might result from increased substrate availability, calcium mobilization, or release of an endogenous compound to activate the cyclase. (The same problems are inherent in a broken-cell vesicular preparation, an extension of the broken-cell homogenate technique (63)). Whilst total levels of cyclic AMP are usually measured in slices, attempts to gain a measure of synthesis have been carried out using labelled adenine or ATP as precursor (64). The following points summarize the responses of cyclic AMP to neurotransmitters using the incubated respiring slice:

i) Slices, usually $260/\mu m \times 260/\mu m \times 1mm$, require a period of stabilization of 30 - 90 mins in oxygenated buffer to overcome the post-mortem elevation of cyclic AMP (50).

ii) Putative neurotransmitters elevate cyclic AMP by stimulation of receptors for noradrenaline, dopamine, histamine, serotonin, adenosine and acidic amino acid compounds. Using blocking agents it appears that these compounds have independent sites of action, although various combinations may show synergistic responses (65).

iii) The cyclic AMP response is rapid, and on removal of the

stimulating agent cyclic AMP levels return to normal. Restimulation usually gives much smaller responses, probably due to increased activation of phosphodiesterase (66).

iv) Omission of calcium usually enhances responses, since calcium ions inhibit neurotransmitter stimulation of adenylate cyclases (67).

v) Phosphodiesterase inhibitors usually enhance accumulation, though marked differences occur between different compounds.

vi) A wide variation in response is found between different species, and strain differences have been reported (68).

vii) Slices from different brain areas vary in response, usually in parallel with the known distribution of the pathways of the neurotransmitter used for stimulation.

viii) Evidence from slices adds weight to a post- rather than a pre-synaptic distribution of cyclic AMP. Brain slices from animals pre-treated with 6 - hydroxydopamine to destroy pre-synaptic nerve terminals containing catecholamines, show increased cyclic AMP responses elicited by noradrenaline (69).

ix) Incubated brain slices may not only accumulate, but also release cyclic AMP into the medium on stimulation (70)

The cyclic AMP content of brain slices can be elevated several fold by electrical stimulation or depolarizing agents, due to indirect release of adenosine. Low concentrations of this purine base (10-100 μM) cause a 20 - 30 fold increase in incubated brain slices, an effect augmented by biogenic amines and completely antagonized by methyl xanthines (71).

1.4. Cellular localization of the cyclic AMP system in the C.N.S.

Incubated slices, whilst being responsive to neurotransmitters, are complex in that they contain a variety of cell types - neurones,

glia, vascular and connective tissue cells. In the present discussion, neurotransmitter responses have been assumed to occur in nerve cells, but there is evidence that other cell types may also respond. Tissue culture techniques have been used in an attempt to study responses of a single cell type, in contrast to the heterogenous cell types in the brain slice. The cyclic AMP content of glial tumour lines is increased by catecholamines, histamine and adenosine, acting via specific receptors e.g. (72), whereas neuroblastoma respond primarily to specific prostaglandins and certain other agents (73). β catecholamine receptor agonists whilst increasing intracellular cyclic AMP levels in glioma, have been found to produce large increases in secretion of cyclic AMP into the medium; this secretion was probenecid-sensitive (74). It has been questioned whether these glial and neuronal differences might be unreal, and possibly explained by differences in metabolism. A closer approximation to the 'in vivo' situation would be anticipated using cell populations from normal fetal brains in culture, containing relatively normal proportions of neurones and glia. These cultures accumulate cyclic AMP in response to catecholamines in a similar fashion to brain slices, with a 5 - 10 fold increase in cyclic AMP levels (75). However, catecholamine responses in fetal cells grown in monolayer culture which are selectively enriched in glial cells, show greater than 100 fold increase in response to noradrenaline (76).

These observations are taken to suggest that the catecholamine response 'in vivo' might occur in glial cells. Great care must be taken however, in extrapolating responses from culture to the living brain, as cells in culture may have responses which are

affected by the experimental culturing technique.

'In vivo', destruction of neurones with kainic acid injection has been found to decrease a number of receptor-linked cyclic AMP systems in striatum. However, some receptor systems were unaffected or responses even enhanced, perhaps suggesting a glial localization (77). In determining whether glial cells have a role in the function of cyclic AMP in the C.N.S. it is of interest that adenylate cyclase, phosphodiesterase, cyclic AMP-dependent protein kinase and the calcium-dependent regulator have both a neuronal and a glial distribution (78, 79). A physiological function of cyclic AMP in glia may be in controlling glycogen breakdown to glucose (77).

Homogenates of brain capillaries have been found to have histamine-sensitive adenylate cyclases, with responses antagonized by both H_1 and H_2 blockers. Joo et al (80) have proposed that cyclic AMP mediates the regulation of permeability in brain capillaries, and observed increased capillary permeability with dibutyryl cyclic AMP, using ferritin as a permeability marker at the electron microscope level.

1.5. Electrophysiological studies.

Having considered 'in vivo' and 'in vitro' biochemical techniques for studying the effects of neurotransmitters on cyclic AMP, an 'in vivo' electrophysiological system will now be discussed in detail. The role of cyclic AMP in mediating the electrophysiological responses of noradrenaline in the locus coereleus-cerebellar Purkinje cell pathway has been extensively studied, principally by Bloom's group. This pathway is ideally suited to '2nd messenger'

analysis since:

i) The pathway has been well characterized as noradrenergic.

ii) Electrophysiological responses are clearly defined in the large Purkinje cells, on stimulation of this pathway.

iii) A noradrenaline-sensitive adenylate cyclase has been biochemically well characterized in cerebellum.

Noradrenaline-containing axons in the cerebellar cortex were shown to be visible at the light microscope level using the Falk-Hillarp fluorescence histochemical technique (81). Employing a high sensitivity electron microscopic technique with uptake of $[^3H]$ noradrenaline, a large number of terminals were found to synapse on the dendrites of the large Purkinje cells which control output from the cerebellum (81). The source of these noradrenergic terminals was found in the locus coereleus. When stimulating electrodes were placed in locus coereleus and recording microelectrodes in Purkinje cells, delayed and long duration inhibition resulted. This type of inhibitory response could only be observed following stimulation of locus coereleus (82). Inhibition which resulted from stimulating certain other areas of the brain stem, could be clearly distinguished by its fast latency, short duration characteristics. Several different approaches were used to provide evidence that the locus coereleus-cerebellar Purkinje cell pathway was noradrenergic, and mediated via cyclic AMP (83, 84, 85).

i) Iontophoretic application of noradrenaline or cyclic AMP slowed the spontaneous firing rate of Purkinje cells by the same type of inhibition as stimulation of locus coereleus. Whilst phosphodiesterase inhibitors had no effect on their own,

this form of inhibition, by stimulation, or iontophoretic cyclic AMP, was potentiated in the presence of phosphodiesterase inhibitors. β receptor blockers and prostaglandins E1 and E2 (β receptor blockers in the periphery), prevented the inhibition by stimulation or iontophoretic noradrenaline, but not cyclic AMP.

ii) Chronic pre-treatment of rats with reserpine (to inhibit catecholamine uptake) or α-methyl paratyrosine (to inhibit catecholamine synthesis), abolished the inhibitory response of Purkinje cells to locus coereleus stimulation or inhibition by iontophoretic noradrenaline, but not by cyclic AMP. Pre-treatment with intracisternal 6-hydroxydopamine to destroy catecholamine neurones, prevented the inhibition by locus coereleus stimulation.

Other studies have demonstrated that cyclic AMP may mediate noradrenergic responses in the system. For example, incubated slices of cerebellum increase their cyclic AMP content when exposed to noradrenaline via activation of β receptors (70) and a specific noradrenaline-sensitive adenylate cyclase is found in cerebellar homogenates (6).

Direct evidence has been presented for the presence of β receptors on Purkinje cells of rat cerebellum using a specific fluorescent ligand (86) but the validity of this technique has been recently questioned (87).

It should be noted that difficulties were experienced by a number of groups in repeating the electrophysiological studies of Bloom's group. These now appear to have been explained by technical

difficulties in iontophoretic application of cyclic AMP (88). In an attempt to circumvent iontophoretic problems Gähwiler (89) studied the actions of noradrenaline and cyclic AMP on firing rates in Purkinje cells from explants of rat cerebellum. Whilst cyclic AMP had little effect, high concentrations of noradrenaline and phosphodiesterase inhibited firing. Evidence suggesting that cyclic AMP mediated noradrenergic inhibition came from the observation that phosphodiesterase inhibitors enhanced the the inhibition by noradrenaline and cyclic AMP. Whilst phosphodiesterase activity was probably very high in this system, thus preventing clear cyclic AMP inhibitory responses, the results support Bloom's data.

Indirect evidence 'in vivo', suggested that the cyclic AMP - dependent protein kinase mediated changes in post-synaptic potential in the Purkinje cell membrane. Siggins et al (90) showed that the ability of cyclic AMP and its derivatives to activate a bovine brain protein kinase closely correlated with their ability to depress Purkinje cell discharge.

Many of the studies described for the locus coereleus-cerebellar Purkinje cell pathway have now been carried out on the noradrenergic projection from locus coereleus to the hippocampal pyramidal cells, with similar results (91). Noradrenaline inhibition in the C.N.S. may therefore be generally mediated via cyclic AMP. Electrophysiology and further biochemical studies on slices have added to the suggestion from dopamine-sensitive adenylate cyclase in homogenates, that the post-synaptic actions of this transmitter are also mediated by cyclic AMP. Clearly, similar electrophysiological studies on other defined neurotransmitter systems are required.

1.6. Extension of the classical 'second messenger' role of cyclic AMP

If cyclic AMP mediates post-synaptic potentials in response to neurotransmitters released from adjacent pre-synaptic terminals, restoration of potential to its pre-excitatory phase should occur with removal of cyclic AMP in a very short time course.

Why then:

i) Are cyclic AMP levels elevated in slices for periods from 10 - 30 minutes, upon neurotransmitter or brief electrical stimulation (71, 92) i.e. 10^4 or 10^5 times longer than the duration of inhibitory or excitatory post-synaptic potentials?

ii) Do sub-cellular fractionation studies show cyclic AMP unbound within synaptosomes for 6 - 10 minutes after electrical stimulation (93)?

In framing these questions, McIlwain (94) has suggested that cyclic AMP is transported to other brain areas by diffusion or active transport to influence electrical activity there, after formation at a specific post-synaptic site. In extending the'second messenger' role of cyclic AMP, McIlwain has proposed that this nucleotide may have a role to play in memory. Evidence in favour of movement of cyclic AMP comes from the demonstration of translocation of adenine nucleotides, including cyclic AMP. Schubert et al (95) have shown that electrical stimulation of specific axonal tracts in the C.N.S. can increase the transfer of radioactive compounds derived from $\left[{}^3H\right]$ adenosine, to post-synaptic sites. If cyclic AMP does indeed translocate, this may occur via microtubules. A link between microtubules and cyclic AMP was first found by Goodman et al (96) who showed that isolated microtubular proteins contained a cyclic AMP-dependent protein kinase, and that tubulin, the major

component of microtubules, was phosphorylated by cyclic AMP 'in vitro'. Subsequent work by Greengards group (34) has shown not only phosphorylation of tubulin, but also of microtubule-associated proteins which may have a function in microtubule assembly.

Regulation of neuronal microtubules via cyclic AMP may be responsible for the role of this nucleotide in a variety of other events besides mediating post-synaptic potentials e.g. growth and intracellular transport of various substances including enzymes.

1.7. Pre-synaptic role of cyclic AMP in the C.N.S.

Pre-synaptic regulation of transmitter release has been studied mostly in the adrenal medulla and superior cervical ganglion, but the following example considers pre-synaptic control in dopamine neurones in the C.N.S.

Electrical stimulation of nigro-striatal dopaminergic neurones results in an increase in the formation of labelled dopamine from exogenous tyrosine, through kinetic activation of the rate-limiting enzyme tyrosine hydroxylase, without synthesis of new enzyme (97). Dibutyryl cyclic AMP, the lipid soluble analogue of cyclic AMP, has a similar specific effect in synaptosomal fractions of striatal homogenates (98). This analogue also increases the formation of dopamine from labelled tyrosine in slices of rat striatum (99). Further studies have suggested that the effects of cyclic AMP on tyrosine hydroxylase might be mediated through the activation of a pre-synaptic cyclic AMP-dependent protein kinase.

It has been found that the addition of cyclic AMP-dependent protein kinase to hippocampal soluble fractions results in a several fold increase in tyrosine hydroxylase activity which is cyclic AMP-dependent. Substances known to inhibit the kinase, such as inhibitory modulator, or adenosine, antagonize this activation (100).

Pre-synaptic in addition to post-synaptic cyclic AMP systems, may therefore be operative in certain neurones in the C.N.S.

1.8. Conclusion

Considerable evidence suggests that cyclic AMP mediates the post-synaptic effects of dopamine, noradrenaline and probably other putative neurotransmitters at certain synapses in the C.N.S., via specific protein phosphorylation. Whilst mediation of rapid post-synaptic potentials in determining communication between nerve cells has been studied in most detail, it is clear that cyclic AMP has a variety of other functions not only in neurones, but also in other cell types, and mediates events of longer time course, e.g. receptor sensitivity, protein synthesis, and growth.

The chain of biochemical events in the cyclic AMP system between stimulus and response, together with compartmentalization of components within the cell, enables this ubiquitous molecule to exert its variety of effects.

2. CYCLIC GMP AND ITS ROLE IN THE CENTRAL NERVOUS SYSTEM - LITERATURE REVIEW.

Discovered as a natural constituent of rat urine in 1963 by

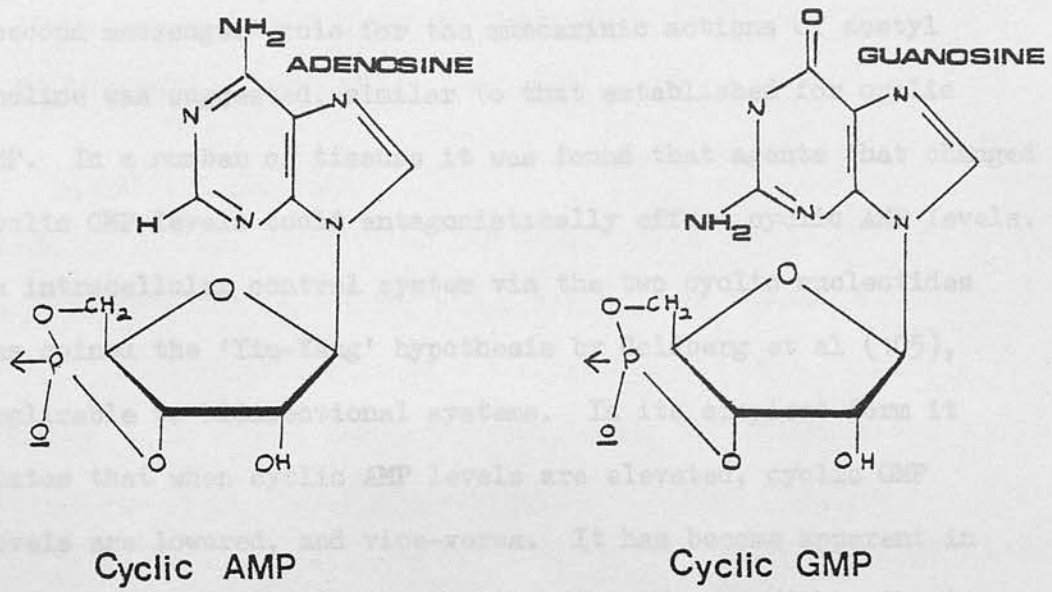


Figure 3 Structure of cyclic AMP and cyclic GMP.

Ashman et al (101), cyclic GMP has a similar structure to cyclic AMP, differing only in the groups and their positions on the purine nucleus (see Figure 3).

The levels of cyclic GMP in tissues are generally much lower than for cyclic AMP, but very high levels, of the same order of cyclic AMP, are found in the C.N.S. in the cerebellum (102, 103). In the search for a physiological function for the nucleotide, George et al (104) in 1970 showed that acetyl choline rapidly elevated cyclic GMP levels in heart, in parallel with negative inotropic, but not chronotropic events.

Together with subsequent observations in different tissues, a 'second messenger' role for the muscarinic actions of acetyl choline was suggested, similar to that established for cyclic AMP. In a number of tissues it was found that agents that changed cyclic GMP levels could antagonistically effect cyclic AMP levels. An intracellular control system via the two cyclic nucleotides was coined the 'Yin-Yang' hypothesis by Goldberg et al (105), applicable to bidirectional systems. In its simplest form it states that when cyclic AMP levels are elevated, cyclic GMP levels are lowered, and vice-versa. It has become apparent in recent years that in certain systems the 'Yin-Yang' hypothesis explains the observations well, whilst in others it is totally inapplicable.

It is also a matter of dispute as to whether cyclic GMP can be regarded as a true 'second messenger'. To quote from a recent extensive review on cyclic GMP by Goldberg:

'.....Attempts to deal with the findings (on cyclic GMP) within the framework of the cyclic AMP 'second messenger' models are often futile, although in some cases a reasonable fit can be made' (106).

The main difficulty in evaluating cyclic GMP as a neurotransmitter or hormonal 'second messenger' has been in showing activation of the cyclic GMP synthesizing enzyme (guanylate cyclase) 'in vitro' by these agents. This contrasts with adenylate cyclase which, as a general rule, is activated by hormones or neurotransmitters in broken-cell homogenates. Most of the reports which have shown stimulation of guanylate cyclase by these agents, have

not been confirmed. In some instances effects have been attributed to contaminants in the hormonal preparation (107) or non-enzymatic formation of cyclic GMP (108).

2.1. Guanylate cyclase.

Guanylate cyclase, catalysing the formation of cyclic GMP from the substrate GTP is, unlike adenylate cyclase, found in both soluble and particulate forms - the particulate form being detected in plasma membrane, endoplasmic reticulum, mitochondria and nucleus (109, 110). The distribution varies from being totally soluble in platelets (111) to totally particulate in sea-urchin sperm (112). Generally however, comparable activity is found distributed between both forms. It has been argued that the soluble form might be an artefact of homogenization deriving from a totally particulate fraction. This now seems unlikely however, since important physico-chemical differences have been found between the two forms, and recently it has been shown that soluble and particulate guanylate cyclase are antigenically discrete (113). It is interesting that the enzyme exists in two forms. For a 'second messenger' role of cyclic GMP the enzyme should be membrane bound to mediate the intracellular response resulting from the extracellular 'first message'. A soluble distribution would be expected if the hormone or neurotransmitter diffused through the membrane like steroid hormones, or if the enzyme had a general role in mediating intracellular processes.

Guanylate cyclase shows an absolute requirement for divalent cations, the substrate assumed to be the divalent cation-GTP complex. In general, the particulate enzyme requires two- to five-fold higher substrate concentrations than the soluble enzyme.

Whilst early studies demonstrated that Mn^{2+} was required in high concentration for activation of both forms of the cyclase it now seems likely that when the enzyme is stimulated, physiological concentrations of Mg^{2+} or Ca^{2+} may substitute for Mn^{2+} (106).

Two further points may have particular interest physiologically. The soluble enzyme is more sensitive to inhibition by ATP than the particulate form, leading Kimura and Murad (114) to speculate that under normal conditions in the cell, little of the soluble guanylate cyclase activity may be expressed unless the ATP inhibition is overcome. Secondly, calcium ions stimulate the soluble enzyme but inhibit the particulate form.

The relationship between the distribution of guanylate cyclase and cyclic GMP levels is of interest; this relationship may be tissue specific. In the rat kidney for example, cyclic GMP levels in different anatomical regions are best correlated with the particulate activity of the region (115). In the testis, however, increased levels of cyclic GMP are clearly associated with an increase in the soluble activity (116).

Whilst a number of reports have shown selective modulation of each form of guanylate cyclase 'in vivo' e.g. increased particulate and decreased soluble guanylate cyclase in regenerating liver, fetal liver and hepatoma (117), as mentioned previously, hormone and neurotransmitter stimulation has not been convincingly demonstrated 'in vitro' and may occur indirectly via 'modulators'. Activation of guanylate cyclase has recently been observed with lipids, free

radicals, nucleophilic compounds and substances affecting oxidation and reduction state, effects which may modulate both the soluble and particulate guanylate cyclase.

Studies on platelets were the first to show that the soluble enzyme could be activated by arachidonic and other structurally related fatty acids (118). Subsequent studies have shown modulation of both forms of the cyclase by a number of fatty acids, in a number of tissues. It is not yet known whether the micromolar levels of fatty acids required for modulation, represent a physiological control system or not.

The first indication that guanylate cyclase might be influenced by an alteration in redox state, was the description by Boehme et al (111) of spontaneous auto-oxidation in air of soluble platelet guanylate cyclase, which was prevented by the reducing agent DTT. Subsequent reports have shown auto-oxidation of the soluble, but not particulate enzyme, from other tissues (e.g. 119).

The oxidized form of ascorbic acid (vitamin C) but not the reduced form, elevates cyclic GMP levels in spleen (106). This effect is probably of little physiological importance though, since it is only seen after cell disruption. It is also of interest in view of redox state effects, that in umbilical artery and kidney, the partial pressure of oxygen together with calcium levels, regulate cyclic GMP levels (120, 121) and that it has recently been reported in cerebellum that cyclic GMP levels may in part be regulated by the partial pressure of oxygen and carbon dioxide (148).

Strongly nucleophilic compounds e.g. sodium azide, and hydroxylamine, have been found by Kimura and Murad (122) unexpectedly to increase

soluble or particulate guanylate cyclase from a number of tissues. In tissues such as cerebral cortex where the soluble enzyme was not found to be activated by azide but the particulate was, the soluble enzyme was activated on addition of a 'non-dialyzable, heat labile, macromolecular factor', isolated from liver and resembling catalase (123). Guanylate cyclase has also been found to be activated by nitrosamines, compounds which readily form free radicals in tissues. The relative effectiveness of nitosamines to activate hepatic guanylate cyclase has been found to correspond with their relative potencies as carcinogens (124). The importance of guanylate cyclase in mediating the toxic effects of not only nitrosamines, but also azide and related compounds with C.N.S. effects, is not yet understood. The gaseous phase of cigarette smoke has been found to activate soluble and particulate guanylate cyclase from a number of tissues, reportedly due to the nitric oxide component of the smoke. An involvement of cyclic GMP in the suspected carcinogenicity of cigarette smoking has been suggested from these results (125). An endogenous activating factor for guanylate cyclase has been found in the synaptosomal fraction of rat brain (126) which may be another indirect mechanism for 'modulating' the enzyme.

Analogous to the cyclic AMP system, the levels of cyclic GMP in a tissue reflect a balance between its rates of synthesis and degradation. Whilst many of the changes in cyclic GMP levels are modulated via the cyclase, the phosphodiesterase, which acts to degrade cyclic GMP. may be an important site of regulation.

2.2. Cyclic GMP phosphodiesterase

Phosphodiesterase, as discussed in section 1.1.C is a group of

isoenzymes. One form is found which hydrolyses cyclic GMP specifically, and evidence that the cyclic GMP and cyclic AMP phosphodiesterases are separate isoenzymes comes from the finding that they are under separate genetic control (127). Control of cyclic GMP phosphodiesterase occurs by a variety of agents, the most important physiologically being calcium ions. The regulation of cyclic nucleotide hydrolysis by calcium might be more directed to the control of cyclic GMP rather than cyclic AMP metabolism since in brain cyclic GMP phosphodiesterase is associated with the calcium-dependent regulator (CDR), and addition of CDR and calcium to brain phosphodiesterase increases the hydrolysis rate of the cyclic GMP phosphodiesterase (44). It is evident that the intense interest being focused on CDR at the moment will give us more information on cyclic nucleotide function in the near future.

A clear example of regulation of cyclic GMP levels via the phosphodiesterase rather than guanylate cyclase, is found in retinal dystrophic diseases. Due to an inherited genetic mutation in mice of the strain C3H/HEJ, all the photoreceptor cells in the retina, whilst forming normally, degenerate during the second and third weeks of post-natal life, causing blindness. Normally the cyclic GMP phosphodiesterase increases about six-fold as the photoreceptors differentiate and mature, but in the diseased retina, the relatively low activity present at birth remains throughout life, causing elevated cyclic GMP levels in the photoreceptor cells (128). Lolley and Farber have proposed that the high cyclic GMP levels cause degeneration of photoreceptors, and therefore blindness. This was born out by their study (129) showing selective and dose-dependent induction of photoreceptor degeneration, by the drug iso-butyl

methyl xanthine (IBMX) using Xenopus eye rudiments in culture. This drug is a potent phosphodiesterase inhibitor and caused accumulation of cyclic GMP in the preparation with increasing concentrations of drug.

Another inherited retinal dystrophy in rats (RCS type) has been found to be associated with alteration of the kinetic characteristics of the cyclic GMP phosphodiesterase affecting the high Km phosphodiesterase (128) and recently, inherited retinal degeneration of Irish setters has been found to be due to the same mechanism (130).

Whilst phosphodiesterase is the only enzymatic method for degrading cyclic GMP, secretion and diffusion may control levels of the nucleotide to some extent. Analogous to cyclic AMP, cyclic GMP is secreted into the cerebro-spinal fluid (CSF), by a probenecid-sensitive mechanism, and CSF cyclic GMP levels are influenced by the systemic injection of drugs (131). Efflux of cyclic GMP as observed from incubated pineal glands, could act to remove cyclic GMP from its site of action, thereby controlling levels (132).

2.3. Cyclic GMP - 'second messenger'?

Before considering a 'second messenger' function for cyclic GMP in the C.N.S., two examples from the periphery will be cited to indicate the controversy in the literature.

In smooth muscle, original observations associated increases in cyclic GMP levels with muscarinic stimulation causing contraction (133), observations in line with the original observations of George et al (104) in heart. Subsequent work on smooth muscle however has dissociated the cyclic GMP increases from contraction and agents

with no effect on contraction,or even those causing relaxation, have been found to increase cyclic GMP levels (133).

Whilst cyclic GMP has always been associated with the muscarinic actions of acetyl choline, Greengard et al (135) have recently reported that cholinergic agonists can increase cyclic GMP levels in striated muscle by acting at nicotinic cholinergic receptors.

In the peripheral nervous system, the sympathetic ganglion has been extensively studied as a system where cyclic AMP and cyclic GMP show opposing electrophysiological responses (136, 137). Pre-ganglionic fibre stimulation causes release of dopamine from inhibitory inter-neurones synapsing on the post-ganglionic neurones, and release of acetyl choline that acts directly on these post-ganglionic neurones. Amongst the post-synaptic potentials resulting from pre-ganglionic fibre stimulation, cyclic AMP is considered to mediate the slow excitatory post-synaptic potential (slow EPSP) elicited by dopamine, whilst cyclic GMP mediates the slow inhibitory post-synaptic potential (slow IPSP) elicited by acetyl choline. The integrative capacity of the ganglion is believed to be due to the long duration of these clearly defined potentials. Recent work, however, has cast doubt on the specificity of the cyclic nucleotides in acting as 'second messengers' mediating the neurotransmitter and electro-physiological responses. Busis et al (138) using the sucrose gap technique, whilst confirming the elevation of the cyclic nucleotides by dopamine and acetyl choline, were unable to demonstrate slow post-synaptic potentials by applying cyclic AMP, cyclic GMP or their more permeable dibutyryl analogues. The classical 'second messenger' test of using a phosphodiesterase inhibitor to prevent cyclic nucleotide degradation, instead of extending slow post-synaptic/

potentials, produced complex electrophysiological responses with theophylline, markedly different from those obtained with another phosphodiesterase inhibitor, papaverine.

In slices of bovine superior cervical ganglion, depolarizing agents have been found to elevate cyclic GMP levels; these observations might be explained by release of acetyl choline. However, sodium azide has also been found to elevate cyclic GMP levels, independent of calcium, in the ganglion, raising further questions as to the specificity of cyclic GMP as a 'second messenger' for acetyl choline in this tissue (139).

In another peripheral nervous tissue, Horn and Mcaffee (140) have shown hormonal changes in cyclic AMP and cyclic GMP levels, in frog sciatic nerve. The ability of nervous tissue to respond to hormones with changes in cyclic nucleotides in a non-synaptic preparation, without effects on excitability, suggests caution in attributing cyclic nucleotide changes in nerve tissue to synaptic events.

It is becoming increasingly clear in a number of systems, that whilst specific receptor stimulation can cause increases in cyclic GMP levels, increases in the nucleotide levels are not necessarily responsible for initiating the physiological effect, and therefore acting as true 'second messengers'. It seems more likely that cyclic GMP increases are somehow involved indirectly, in linking the actions of neurotransmitters or hormones with their physiological effects, perhaps by mediating ion fluxes.

2.4. Cyclic GMP in the C.N.S.

Is cyclic GMP a 'second messenger' to acetyl choline in the central nervous system?

Since very high levels of cyclic GMP are found in the cerebellum (103), extensive studies have been carried out in this brain area where cyclic GMP might be expected to have an important function. The distribution of neither the soluble, nor particulate guanylate cyclase, nor the muscarinic receptor, show any correlation with cyclic GMP levels in different brain areas, which might have been expected if cyclic GMP was a 'second messenger' to acetyl choline (141). Following the work of George et al (104) on acetyl choline and cyclic GMP in heart however, Ferrendelli et al (142) showed that the centrally acting cholinergic agent, oxotremorine, increased cyclic GMP levels in mouse cerebellum and cortex. The time of maximal tremor produced by the drug, coincided with maximal cyclic GMP increase, tremor and elevation in nucleotide level being blocked by the anti-cholinergic drug atropine. This result suggested that oxotremorine was acting via central muscarinic receptors to cause tremor. The cerebellar cyclic GMP increase must have been an indirect effect, since cholinergic receptors have not been found in this brain area. Goldberg et al (143) showed that elevation of acetyl choline levels 'in situ' increased cyclic GMP levels, since administration of malaoxone, a centrally acting cholinesterase inhibitor, elevated acetyl choline levels in the C.N.S. together with cyclic GMP.

It has been subsequently found, however, that a variety of agents and conditions, assumed to act on a variety of neurotransmitter systems can modify cyclic GMP levels 'in vivo' e.g. amphetamine,

chlorpromazine, reserpine, halothane and stress (144, 145, 146, 147). Post-mortem decapitation effects are also found with cyclic GMP as previously described for cyclic AMP. Whilst similar reservations hold for cyclic AMP as for cyclic GMP in interpreting 'in vivo' data, it is possible to generalize that drugs or conditions that increase general neuronal activity e.g. tremorogenic drugs and convulsants, increase cyclic GMP centrally, whilst decreased levels are associated with depression of neuronal activity e.g. after administration of morphine and ethanol (55).

Recent data suggests that decreases in cerebellar cyclic GMP caused by drugs may be secondary to decreased motor function or respiratory depression produced by the drugs (148). The 'in vivo' data suggests, therefore, that a basic neuronal mechanism might underly changes in cyclic GMP levels in the C.N.S.

2.5. 'In vitro' biochemical studies.

The inability of neurotransmitters to stimulate guanylate cyclase in broken-cell homogenates has led to a large number of studies on the respiring slice. A number of putative neurotransmitters have been shown to elevate cyclic GMP levels in slices e.g. acetyl choline, glutamate, GABA, glycine, noradrenaline, histamine and enkephalin. There appears to be a marked species and brain area variation in the response (147, 149).

Recently, Ferrendelli's group have demonstrated that far greater elevations can occur in brain slices exposed to depolarizing agents (150, 151). Exposure to high levels of K^+ (17-120 mM), and depolarizing agents acting by different mechanisms (veratridine ouabain, glutamate) caused a 30-50 fold increase in slices of mouse

cerebellum, which was prevented by membrane stabilizing agents e.g. local anaesthetics. Depolarization causes a number of changes in biochemical and functional processes e.g. change in cell membrane permeability, increased cellular respiration and augmented neurotransmitter release. Examining the effect of divalent cations on the depolarization-induced increases in cyclic GMP, Ferrendelli's group demonstrated that all the depolarizing agents required calcium ions in order to elevate cyclic GMP levels. Furthermore, the divalent cation requirement for elevations in cyclic GMP paralleled that for neurotransmitter release at the neuromuscular junction and other synapses. This suggested that cyclic GMP might be involved in neurotransmitter release as suggested in the pineal gland, for regulation of noradrenaline release (152).

A large number of putative neurotransmitter agonists and antagonists were then examined in incubated brain slices, to determine the effect of released neurotransmitters on cyclic GMP levels. A number of observations suggested that depolarization-induced increases in cyclic GMP were not due to release, or action, of these putative neurotransmitters, or to presently unidentified neurotransmitters since:

i) the extent of the increase was far less than those produced by depolarizing agents,

ii) whilst K^+ ions were found to increase tissue levels of cyclic GMP in slices of a number of species, and in a number of different brain areas, the neurotransmitter increases showed both species and tissue selectivity.

iii) The K^+ induced increases in cyclic GMP were not blocked by any putative neurotransmitter antagonist.

iv) Acid extracts of rapidly frozen mouse cerebellum, or aqueous extracts of boiled mouse cerebellum had no effect on cyclic GMP levels in mouse cerebellum.

Having discussed variability in response between species and brain area, recent data (153) has shown a marked age-dependency in the response of rat cerebellar slices to depolarization induced by glutamate. The supersensitive response was found to peak post-natally at day 10, causing greater than a 200-fold increase in cyclic GMP over basal levels, contrasting with approximately a 2-fold increase in adult slices. Decreased responses of cerebellar slices to accumulate cyclic GMP after stimulation by kainic acid, have also been observed in rats between 3 months and 2 years old; basal levels were also reduced (154).

Depolarization induced increases in cyclic AMP in brain slices, assumed to be due to release of intracellular adenosine, markedly contrast with cyclic GMP increases, by dose and time-dependent differences in response (150).

It is of interest, however, that a recent report shows that adenosine can increase cyclic GMP levels in guinea-pig cerebellar slices 3 - 4 fold, dependent upon calcium; AMP, ADP and ATP produced similar increases (155), whilst adenine and other nucleotides had no effect. Perhaps the most important difference between cyclic AMP and cyclic GMP responses in slices is that the latter are dependent upon calcium ions. The importance of calcium for this response is born out by the observation that cyclic GMP

levels are increased in brain slices incubated with the calcium ionophore, A23187, which acts as a mobile carrier, to transport calcium across cell membranes (150). Calcium ion flux may underly the cyclic GMP increases observed with depolarizing agents, by regulating the calcium-dependent soluble guanylate cyclase. The importance of calcium ions for maintaining basal cyclic GMP levels and eliciting responses in a wide variety of tissues outside the C.N.S., has led Schultz et al (156) to propose that calcium may act as the intracellular 'second messenger' for muscarinic stimulation of guanylate cyclase. In this respect it may be of importance that drugs which decrease 'neuronal activity' can deplete regional brain calcium (157).

Calcium-dependent elevation of cyclic GMP in slices is probably due to an action on guanylate cyclase rather than the phosphodiesterase, inferred from studies where inhibitors of phosphodiesterase were used (151). Since neurotransmitter activation of guanylate cyclase has not been convincingly demonstrated 'in vitro', calcium-dependent elevations of cyclic GMP may result from increasing availability of calcium ions to the soluble enzyme. Kimura and Murad (122) have demonstrated calcium-independent elevation of cyclic GMP levels in brain slices using sodium azide, resulting from stimulation of the particulate guanylate cyclase.

The 'in vivo' and 'in vitro' data presented suggest that neurotransmitters probably influence cyclic GMP indirectly by a basic mechanism such as changing calcium ion flux or effecting modulators.

2.6. Electrophysiological studies

Electrophysiological data is not only far more limited than for cyclic AMP, but also conflicting. Stone et al (158) have shown good correlation between the excitatory effects of acetyl choline and cyclic GMP in pyramidal neurones of the rat cerebral cortex. Noradrenaline and cyclic AMP elicited opposing inhibitory responses in these cells, conforming to the 'Yin-Yang' hypothesis of biological regulation described previously. In contrast to these results, a study by Phyllis (159) using extracellular application of acetyl choline onto pyramidal neurones and other neurones caused excitation, whereas cyclic GMP depressed firing rate. An intracellular iontophoretic study of acetyl choline and cyclic GMP in the large spinal motor neurones of the cat, produced different effects of the two substances. Cyclic GMP decreased input resistance, whilst acetyl choline was found to increase it (160). Again in contrast, intracellular studies by Woody (161) in awake cats in the coronal pericruciate cortex, showed that cyclic GMP increased input resistance in about half the cells tested. Similar responses were elicited in the same cells with acetyl choline, which were blocked with atropine. Iontophoresis of acetyl choline or cyclic GMP to these neurones, held under repeated discharge by electrical stimulation, caused long lasting increased resistance (up to 1½ hours in unanaesthetized awake cats).

Woody (161) suggests that the persistent increased membrane resistance may mediate learned behaviour. This is clearly in line with McIlwain's extended 'second messenger' hypothesis for cyclic nucleotides (94).

Whilst the electrophysiological data on cyclic GMP in the C.N.S. is controversial, it is clear at least that the nucleotide is associated with changes in neuronal excitability in the C.N.S.

2.7. Cellular localization of the cyclic GMP system in the C.N.S.

A neuronal rather than glial distribution of guanylate cyclase is inferred from the following data:

i) The sub-cellular distribution of soluble guanylate cyclase resembles that of other enzymes considered as neuronal markers e.g. glutamate decarboxylase and tyrosine hydroxylase, but differs from that of soluble non-specific cytoplasmic markers such as lactate dehydrogenase and the two glial soluble markers, S-100 protein and butyryl cholinesterase (162).

ii) Studies of chick embryo brain cells in culture show that guanylate cyclase activity is always higher in cultures with a significantly greater ratio of neurones to glia (163).

iii) In glial or meningeal cell cultures, guanylate cyclase is below the limit of detection (163).

The above data suggests that guanylate cyclase is mainly if not exclusively, concentrated within neurones.

Experiments with neuronal and glial cells in culture have been carried out to determine whether only neuronal or both cell types, respond to stimulation. As discussed with this experimental approach for cyclic AMP, care should be taken in extrapolation of results to the 'in vivo' situation. In the presence of the phosphodiesterase inhibitor IBMX, cells of the mouse neuroblastoma line NIE 115 were found to respond to muscarinic stimulation with

a 200-fold increase in cyclic GMP, which was blocked by atropine and was dependent upon calcium. The importance of calcium ions in eliciting the response in this nerve cell line, was shown by the large increases which were generated using the calcium ionophore A 23187, in the presence of 1mM Ca^{2+}, a response which could not be blocked by atropine (141). Elevations in cyclic GMP in neuroblastoma have also been observed with non-cholinergic agents e.g. histamine has been found to increase cyclic GMP levels in mouse neuroblastoma via H_1 receptors (164).

Using C6 rat glial cells in culture, catecholamines have been found to elevate cyclic GMP levels, acting via a β receptor. Carbamylcholine, a cholinergic agonist, was found to decrease cyclic GMP levels, a response partially antagonized by hexamethonium, suggesting nicotinic characteristics (165). In human astrocytoma cells however, carbamylcholine was found to increase cyclic GMP levels (166).

A number of neurotransmitters were found to increase cyclic GMP levels in a vascular endothelial cell line. The cyclic GMP response to noradrenaline was blocked by the α antagonist phentolamine, whilst atropine blocked the response to acetyl choline (167).

An interesting observation on neuronal and glial cyclic GMP has come from a study by Taylor et al (168) who micro-dissected a large number of insect nerve cords to prepare neuronal and glial enriched fractions. Cyclic GMP was concentrated in the glial fractions, levels being significantly higher than for cyclic AMP which had similar levels in both fractions.

2.8. Control of cyclic GMP levels in the cerebellum.

The cerebellum, with very high levels of cyclic GMP, and highly responsive to a large number of agents and conditions, contains a number of different neuronal and glial cell types. In an attempt to determine which cells contain cyclic GMP and respond to stimulation, Ferrendelli et al (169) have employed micro-dissection of the clearly defined layers of the cerebellar cortex, coupled with a highly sensitive radioimmunoassay for cyclic GMP. After drug treatment, most of the changes in cyclic GMP levels were found in the molecular layer which contains predominantly fibres and synapses; the remainder of the changes occurred in the granule cell layer. The lack of a significant pool of the nucleotide in the Purkinje cell layer contrasted with the findings that mutant ataxic mice lacking Purkinje cells, had significantly reduced cerebellar cyclic GMP levels, suggesting that the nucleotide was concentrated in these cells (170). The molecular layer contains Purkinje cell dendrites however, which might make the observations compatible. Subsequent 'in vitro' data using these mutant mice has shown that large increases in cyclic GMP can be elicited in cerebellar slices with K^+ depolarization, noradrenaline and kainic acid (171). Evidently, even if there is a Purkinje cell pool of cyclic GMP, it cannot be the only one.

Information is now accumulating that the neural input to the cerebellum controls cyclic GMP levels. The tremorogenic drug harmaline increases cyclic GMP in the cerebellum by stimulating nerve impulse flow in the climbing fibre input (172,173). Specific chemical lesion of the climbing fibres using the drug 3-acetylpyridine abolishes the response to harmaline. The second input via mossy

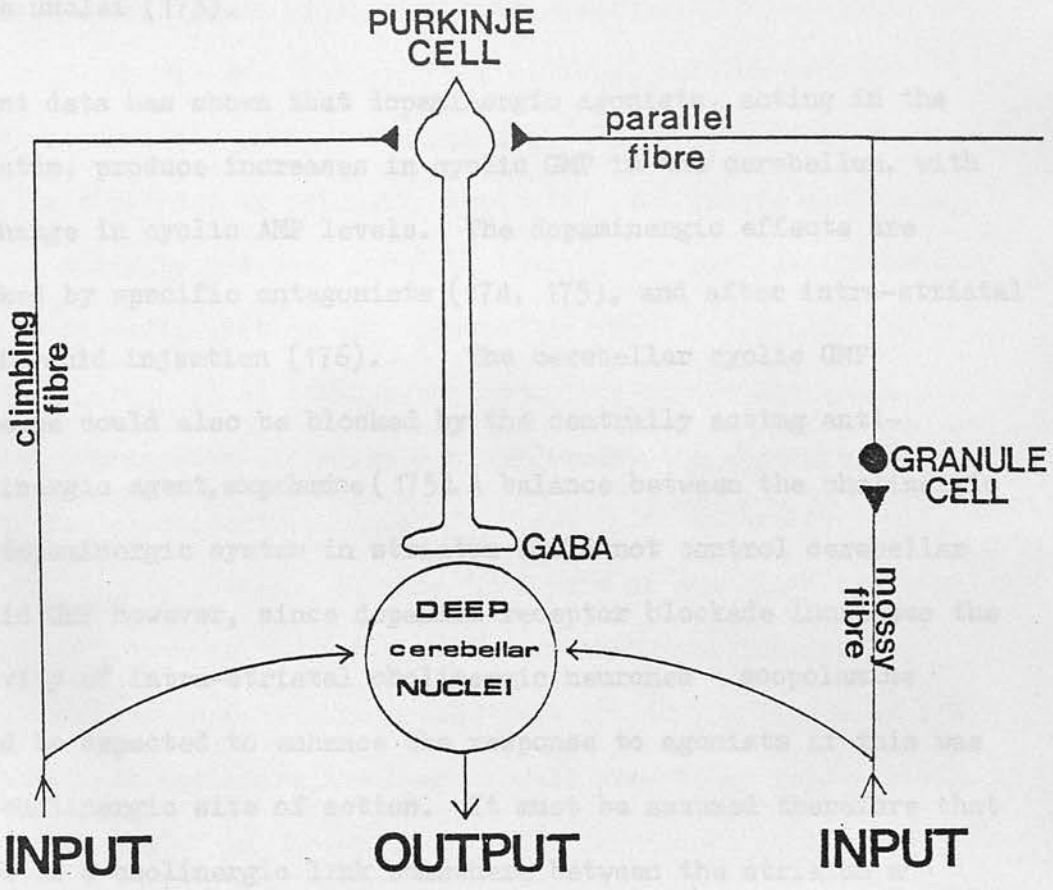


Figure 4 Neuronal systems regulating input and output of information in the cerebellum.

fibres is stimulated by the drug isoniazid; this response is unaffected by climbing fibre lesions (172). The neuronal output from Purkinje cells is inhibitory on neurones in the deep cerebellar nuclei and mediated by γ-aminobutyric acid (GABA). Biggio et al (173) have shown that the cyclic GMP content of these nuclei is decreased following activation of GABA receptors by muscimol and diazepam, and is increased following a reduction

of GABA control caused by isoniazid. Climbing fibre lesions have no effect, neither does harmaline, on the cyclic GMP content of these nuclei (173).

Recent data has shown that dopaminergic agonists, acting in the striatum, produce increases in cyclic GMP in the cerebellum, with no change in cyclic AMP levels. The dopaminergic effects are blocked by specific antagonists (174, 175), and after intra-striatal kainic acid injection (176). The cerebellar cyclic GMP increase could also be blocked by the centrally acting anti-cholinergic agent, scopolamine (175). A balance between the cholinergic and dopaminergic system in striatum could not control cerebellar cyclic GMP however, since dopamine receptor blockade increases the activity of intra-striatal cholinergic neurones - scopolamine would be expected to enhance the response to agonists if this was the cholinergic site of action. It must be assumed therefore that there is a cholinergic link somewhere between the striatum and cerebellum.

Two possible caudato-cerebellar pathways may exist. One pathway passing through the red nucleus and mediating activation of mossy fibre input, and the other relaying in the substantia nigra and inferior olive, and mediating the activation of the climbing fibre input to Purkinje cells. Experiments are being carried out in a number of laboratories on the specific pathways mediating cerebellar cyclic GMP levels.

The role of cyclic GMP in the C.N.S. is clearly complex and not well understood at present. Attempting to summarize the 'in vivo' and 'in vitro' data, it seems that neuronal activity regulates

cyclic GMP levels perhaps by mediating calcium ion fluxes. The data previously described demonstrates that cerebellar cyclic GMP levels are influenced by neural input. Whilst this response may occur through activation of the soluble, calcium-dependent guanylate cyclase, what of the function of the particulate form which is inhibited by calcium? Increased particulate guanylate cyclase activity has been found in liver after partial hepatectomy, a model of proliferating cell growth 'in vivo' (117). In the C.N.S. Kumakura et al (177) have shown that in certain brain tumours (meningomas and oligo-dendrogliomas) the apparent Km of GTP for particulate guanylate cyclase is considerably lower than for normal cortex. Perhaps this form of guanylate cyclase regulates growth in the C.N.S., since a number of reports for non-nervous tissues have suggested that cyclic GMP is one of the intracellular mediators involved in cell growth and cell division (e.g.178, 179). In the C.N.S., cyclic GMP levels increase post-natally with marked regional variation. Levels are very low at birth and up to 12 days, but between this time and adulthood, cerebellar levels show a particularly marked increase (103, 180). Guanylate cyclase activity shows an opposite pattern however, immature rats having very high activity which decreases towards adulthood (180).

2.9. Cyclic GMP-dependent protein kinase and protein phosphorylation

In a similar manner to cyclic AMP, cyclic GMP is proposed to exert its physiological effects by binding to naturally occurring kinases, which have recently been found with high specificity for cyclic GMP as substrate (181, 55, 106). Difficulty has been experienced in showing dissociation of the regulatory and catalytic units of the

cyclic GMP-dependent kinase in the past, suggesting that dissociation may not be required for activation. Kuo (182) has recently demonstrated, however, that a modulator protein is essential to demonstrate dissociation on cyclic GMP binding.

In the C.N.S. cyclic GMP kinase is mainly distributed in the soluble fraction, the major cyclic GMP binding sites being associated with the cyclic GMP-dependent kinase (181, 183). Studies on the cellular localization of cyclic GMP kinase in the cerebellum using mutant mice with Purkinje cell deficiencies, and 3-acetyl pyridine lesions in normal mice, have shown that the kinase is associated with Purkinje cells and their dendrites (184). It is of interest that similar techniques have shown similar localization of cyclic GMP. An inhibitory modulator of cyclic GMP kinase has been described in the cerebellum (185). Harmaline, which increases cyclic GMP levels in cerebellum, decreased the activity of the inhibitor; both of these biochemical effects were prevented after 3-acetyl pyridine lesion of the climbing fibres. In contrast, diazepam which lowers cyclic GMP levels in cerebellum, increased the activity of the inhibitor by 100%.

In searching for the natural substrates phosphorylated by the cyclic GMP kinase, Greengard's group first showed phosphorylation of two membrane proteins in smooth muscle by cyclic GMP (186). Recently they were able to show phosphorylation of a specific 23,000 M.W. protein in the soluble fraction of cerebellar homogenates (187). Two points are of special interest:

i) That a soluble rather than membrane protein perhaps controlling ion fluxes, should be phosphorylated.

ii) That this phosphorylated protein should be demonstrated in

cerebellar homogenates, but not homogenates of caudate, hippocampus, or cerebral cortex.

Further work is required to determine whether phosphorylation mediates physiological effects of cyclic GMP.

2.10. Conclusion.

The role of cyclic GMP in the C.N.S. is considerably less well understood than for cyclic AMP. It is becoming increasingly apparent that in contrast to the specificity of cyclic AMP 'second messenger' effects, cyclic GMP levels are influenced by a number of common biochemical conditions e.g. ion fluxes and redox state, which probably mediate the 'indirect' effects of neurotransmitters on cyclic GMP. 'In vivo', cyclic GMP levels specifically in the cerebellum, may mediate long-term electrophysiological events by responding to dopaminergic/cholinergic neural input. The soluble and particulate distribution of guanylate cyclase, allowing specialization of effects due to different distribution in the cell, may have very different functions. The particulate form might play a role in affecting growth, whilst the soluble, calcium-dependent enzyme, coupled with the finding that cyclic GMP phosphorylates a soluble protein in cerebellum, might suggest a soluble neuronal system.

3. CYCLIC NUCLEOTIDE IMMUNOHISTOCHEMISTRY

3.1. Principles of immunhistochemistry

Histochemistry aims to localize substances 'in situ' in tissue sections with high specificity and high sensitivity. A substance may be visible under a normal light or ultra-violet light microscope or be rendered visible by means of a specific

dye. Alternatively a dye or radioactive label may be 'tagged' to a molecule which will bind the substance in the tissue section, or a specific chemical reaction be employed to form a coloured reaction product to identify the substance.

Immunohistochemistry is an advanced histological technique employing the high specificity and sensitivity of immunological reactions for localizing molecules as antigens within tissue sections. Antibodies have to be made visible by linking them with 'tags' in such a way that their characteristics for recognizing and binding with antigen are not changed. This 'tagging' was first attempted with azo dyes in 1933 (188), but the low sensitivity of the antibody-dye conjugates, prevented general usage. Hopkins et al (189) tried to use fluorescent dyes with the hope of increasing sensitivity, but the antibody-dye linkage interfered with the interaction with antigen. In 1941-2, Coons et al (190, 191) succeeded for the first time in linking a fluorescent dye with an antibody, and preserving antigen binding, which enabled localization of soluble polysaccharide antigen in tissue sections, from mice infected with pneumococcus. Conjugates of the antibody and the dye fluorescein isocyanate, showed high intensity greenish-yellow fluorescence in high dilution, but the chemical procedures required for labelling, together with the instability of the dye, prevented widespread use of the technique. In 1958 however, Riggs et al (192) synthesized a more stable compound, fluorescein isothiocyanate, which was easily conjugated with antibody. Its widespread commercial availability opened up the field of immunofluorescence to a very large number of investigators. Whilst antibodies are only produced by immunization of animals with large

molecules such as proteins, polysaccharides and nucleic acids, antibodies have now been raised to a large variety of small molecules (haptens) coupled with high molecular weight immunogenic 'carrier' molecules (193). The power of immunofluorescence therefore is that any substance can theoretically be used as an antigen and localized histologically with high sensitivity and specificity. Practically, the technique is simple, but considerations such as the solubility and diffusion of small molecules, and fixation, may limit the applicability of the method. Extension of the original work of Coons to techniques with higher sensitivity and wider applicability e.g. at the ultrastructural level, has made immunohistochemistry the fastest growing histochemical technique for visualization and identification of bacterial, viral and animal tissue antigens, both native and foreign, and in modified form for localization and characterization of serum antibodies in tissues (194).

The original technique, known as the direct technique, employs labelled antibody in a single step to localize the antigen in the tissue section. Fluorescein isothiocyanate is still, today, the most widely used dye for immunofluorescence, showing bright apple-green fluorescence under ultra-violet light. Antibody is simply coupled to the dye under alkaline conditions. In certain tissues where endogenous tissue fluorescence (autofluorescence) is a problem the use of a contrasting coloured dye has often proved useful. Lissamine rhodamine, imparting a red fluorescence is most commonly employed for this purpose. In the direct technique, two or more antibodies may be labelled with different coloured dyes and used to view different antigens in the same tissue section. The other dyes that have been developed for use in immunofluorescence (194)

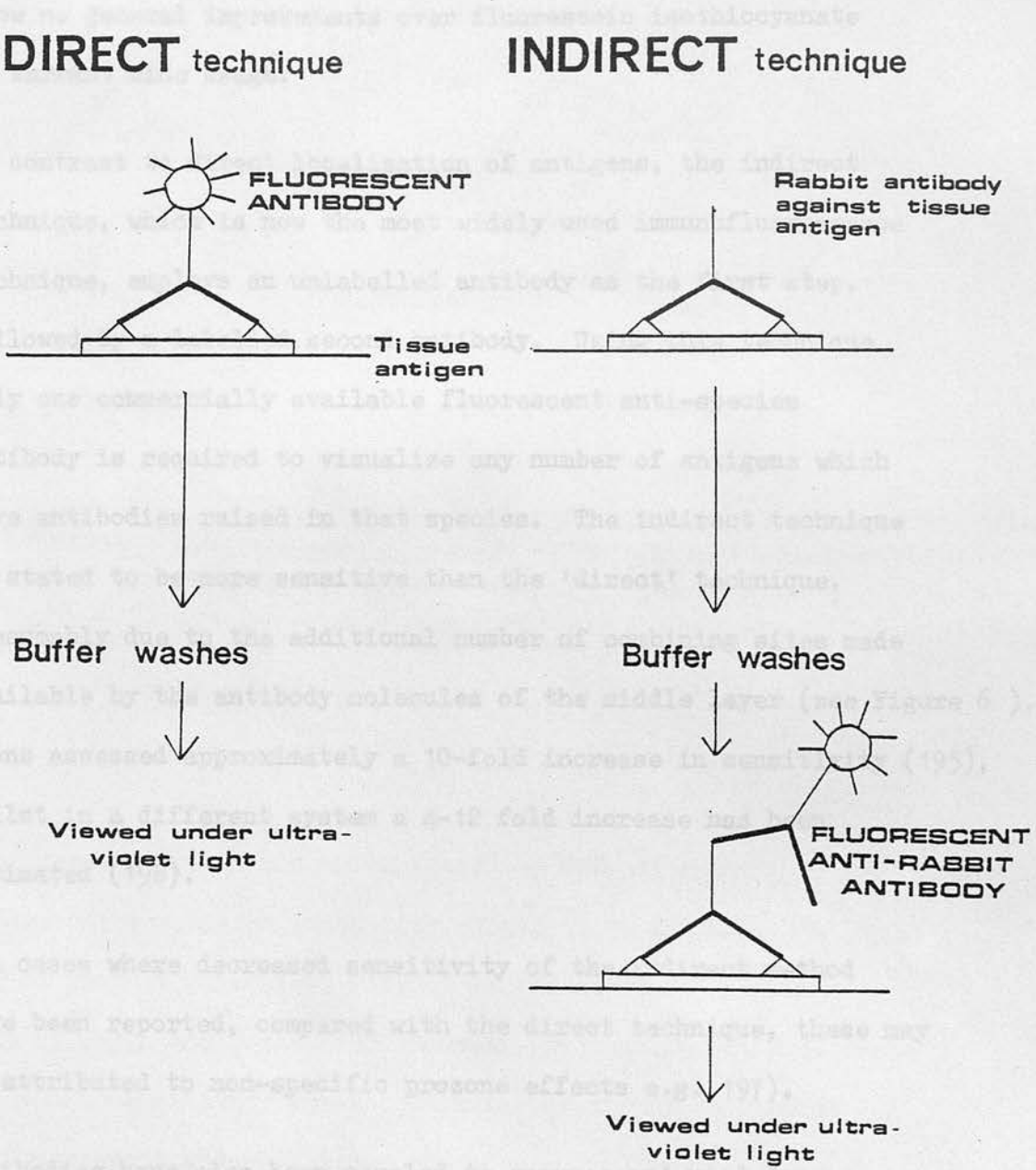


Figure 5 Comparison of the direct and indirect immunofluorescence techniques.

show no general improvements over fluorescein isothiocyanate to warrant wide usage.

In contrast to direct localization of antigens, the indirect technique, which is now the most widely used immunofluorescence technique, employs an unlabelled antibody as the first step, followed by a labelled second antibody. Using this technique only one commercially available fluorescent anti-species antibody is required to visualize any number of antigens which have antibodies raised in that species. The indirect technique is stated to be more sensitive than the 'direct' technique, presumably due to the additional number of combining sites made available by the antibody molecules of the middle layer (see Figure 6). Coons assessed approximately a 10-fold increase in sensitivity (195), whilst in a different system a 4-12 fold increase has been estimated (196).

(In cases where decreased sensitivity of the indirect method have been reported, compared with the direct technique, these may be attributed to non-specific prozone effects e.g. 197).

Antibodies have also been coupled to enzymes and used for immunohistochemistry. Peroxidase has been most widely employed, but other enzyme 'tags' such as cytochrome C and lactate dehydrogenase have also been used. Peroxidase is coupled to antibody using gluteraldehyde or periodate oxidation, and visualized 'in situ' by the formation of a dark brown reaction product, with the specific substrate diaminobenzidine hydrochloride (DAB); the reaction requires hydrogen peroxide (198). Immunoperoxidase has the following advantages over immunofluorescence (198):

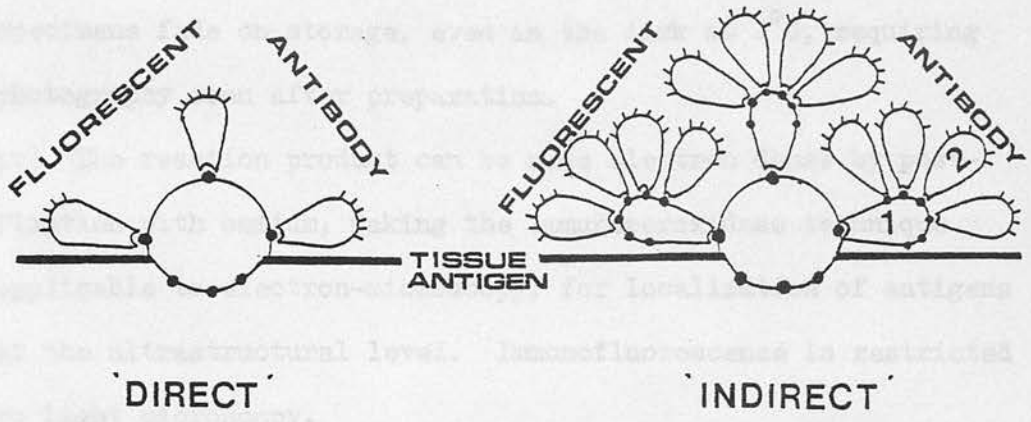


Figure 6 Diagram explaining the increased sensitivity of the indirect immunofluorescence technique in comparison with the direct technique.

i) The sensitivity should be increased by extending the histochemical incubation times with substrate to increase the number of product molecules, without losing specificity. In a study by Petts and Roitt (199) however, no significant difference was found in sensitivity for localization of tissue antibodies using fluorescent and peroxidase 'tags'.

ii) An ordinary light microscope can be used to visualize reaction product, rather than expensive and complex fluorescent microscopes. In situations where extensive microscopy must be carried out by a single observer, light microscopy should give appreciably less eye fatigue compared with fluorescence.

iii) The reaction product is permanent whereas immunofluorescent specimens fade on storage, even in the dark at 4°C, requiring photography soon after preparation.

iv) The reaction product can be made electron dense by post-fixation with osmium, making the immunoperoxidase technique applicable to electron-microscopy, for localization of antigens at the ultrastructural level. Immunofluorescence is restricted to light microscopy.

The disadvantages of immunoperoxidase are:

i) Endogenous peroxidase activity in tissues may interfere with specific staining. Techniques are available for removing endogenous peroxidase however , e.g. 0.074% H Cl in 100% ethanol (200), but care must be taken to ensure that tissue antigenicity is not interfered with.

ii) The reaction product may be a cause of diffusion artefacts, although this is only really a problem at the electron-microscope level (201, 202).

iii) Fluorescence probably has a better resolution, particularly of fine fibres (203).

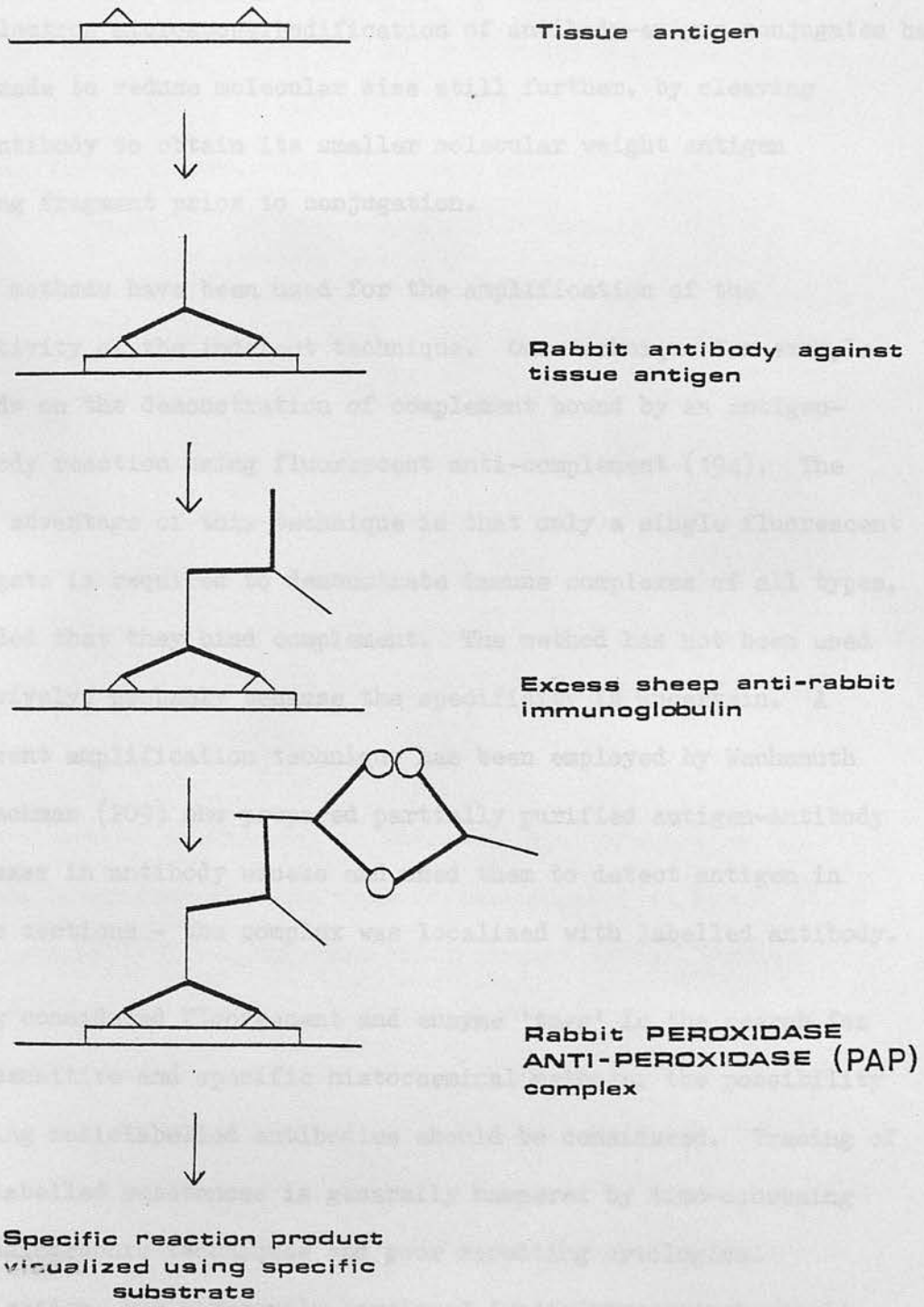
iv) The most serious objection to the immunoperoxidase technique is on safety grounds however, since the DAB substrate may be carcinogenic (204) which has led to its withdrawal from a number of laboratories. Recently, non-carcinogenic compounds have been developed, which appear to be as useful as DAB in localizing peroxidase in axonal transport techniques (205), but information on their use in immunoperoxidase techniques is at present very limited.

The indirect technique has been shown to be more sensitive than the direct technique due to interposition of a second antibody layer. Increasing the number of layers still further has been found to produce greater increases in sensitivity. More sensitive immunoperoxidase techniques were developed simultaneously by Mason (206) and Sternberger et al (207).

The peroxidase anti-peroxidase (PAP) technique (see Figure 7) of Sternberger is now very widely used for localization of antigens particularly at the ultra-structural level, despite the complexities that a multi-layered technique introduces. A soluble complex of peroxidase and antibody to peroxidase, is employed as the third reagent; this PAP complex appears ultrastructurally as black flattened rings, pentagons or spots at the sites of localization of the tissue antigen. The technique is claimed to be 100 - 1000X more sensitive than the indirect fluorescent method at the light microscope level, very low dilutions of first antibody being required. The main advantage, however, is that it is not necessary to conjugate peroxidase to any of the specific antibodies, which may possibly disturb their specificity. This is probably another source of increased sensitivity of the PAP method, since in the indirect immunoperoxidase technique, some antibodies will remain unlabelled with peroxidase, and as these cannot be removed, they may compete with labelled antibodies on the tissue section, thus decreasing the sensitivity of the indirect method.

It is of interest that some reports have claimed no increase or decrease in sensitivity of the PAP technique over the indirect technique. This may be due to inability of PAP molecules, because

Figure 7 The peroxidase anti-peroxidase (PAP) immunohistochemical technique.



of their size, to localize all the antigen at every site (203).

The choice of enzyme marker is dependent to a large extent on its molecular size e.g. peroxidase is much smaller than the marker ferritin, improving its penetration into tissue sections for electron microscopy. Modification of antibody-enzyme conjugates has been made to reduce molecular size still further, by cleaving the antibody to obtain its smaller molecular weight antigen binding fragment prior to conjugation.

Other methods have been used for the amplification of the sensitivity of the indirect technique. One technique for example, depends on the demonstration of complement bound by an antigen-antibody reaction using fluorescent anti-complement (194). The great advantage of this technique is that only a single fluorescent conjugate is required to demonstrate immune complexes of all types, provided that they bind complement. The method has not been used extensively, probably because the specificity is uncertain. A different amplification technique has been employed by Wachsmuth and Lachman (209) who prepared partially purified antigen-antibody complexes in antibody excess and used them to detect antigen in tissue sections - the complex was localized with labelled antibody.

Having considered fluorescent and enzyme 'tags' in the search for more sensitive and specific histochemical methods, the possibility of using radiolabelled antibodies should be considered. Tracing of radiolabelled substances is generally hampered by time-consuming autoradiographic techniques and poor resulting cytological localization, but a recently developed 'radioimmunocytochemical' technique shows that success can be achieved, for high sensitivity

localization of antigen in tissue sections (210).

3.2. Immunohistochemistry - methodological considerations

3.2.A. Tissue preparation:

Light microscope immunhistochemistry is carried out on tissue sections from either frozen blocks as commonly employed, or freeze-dried paraffin or resin-embedded blocks. 'Snap-freezing' of small tissue blocks using isopentane pre-cooled with liquid nitrogen (temperature of isopentane whilst thawing, -160°C.) is a rapid technique which minimizes the formation of intracellular ice crystals detrimental to good histology, and reduces diffusion of soluble substances (194). Direct freezing in liquid nitrogen cannot be used since gas bubbles rapidly surround and insulate the specimen. An alternative freezing method employs solid carbon dioxide and ethanol (-76°C), but this technique is neither as rapid nor as successful as isopentane-liquid nitrogen, particularly for brain tissue where the high lipid content reduces the rate of freezing.

To permit access of antibody to intra-cellular antigen, cells are cut open by cutting sections from frozen or embedded blocks at approximately 4-10/μm.(For immunofluorescence of cell surface antigens which will not be discussed in detail, suspensions may be used for immunfluorescent staining). Frozen sections are cut in a cryostat at approximately -25°C, optimal temperature being dependent on the tissue type and the required thickness of sections to be cut out. Whilst tissue fixation will often be required to fix soluble antigens and obtain good tissue morphology, it is essential that good tissue preservation is coupled with maintenance

of tissue antigenicity. There are only empirical fixation techniques for immunohistochemistry, certain tissue antigens being destroyed by the fixative or the fixation process (dehydration, clearing etc). Experimentation for each antigen in each tissue is therefore required. Immunohistochemistry is frequently carried out on unfixed sections which may be aided by the rapid freezing method, reported by Nairn (194) to involve less need for chemical fixatives. Particular care is required for low molecular weight antigens which may be lost in the tissue water when frozen sections thaw out, or during immunohistochemical processing.

The accessibility of antigen to antibody may be improved by fixation and even detergents. Hartmann (211) has reported that brain sections lipid-extracted with chloroform-methanol, show increased sensitivity for dopamine -β- hydroxylase localization, if low concentrations of Triton X-100, a non-ionic detergent, are added to reagents and washes. For ultrastructural localization of tissue antigens the problem of tissue fixation is considerably more complex since the choice of fixatives is more limited.

3.2.B. Antibody characteristics

Antibody activity is found in the γ -globulin fraction of serum. Five classes of antibody molecules are recognized in most species within this fraction: IgG, IgA, IgM, IgD, IgE. IgG is by far the most important antibody molecule and the γ-globulin fraction is predominantly IgG. An antibody contains a heterogenous population of IgG molecules even when prepared to an antigen which is 100% pure, since most pure antigens contain multiple sites (determinants)

each one giving rise to a specific type of molecule (193).

The amount of specific antibody in an antiserum is termed its titre. Affinity is the measure of the 'strength' of interaction between an antibody and antigen, whilst the term avidity takes into account the heterogeneity in affinity of antibody molecules and represents the net interaction between an antiserum and antigen. Antiserum specificity reflects the molecular 'fit' between antigen and antibody. These antiserum characteristics, titre, avidity and specificity, are affected by a number of immunization factors e.g. inter-species and inter-animal variations, quantity of immunogen for injection, time course between immunizations (see ChapterIII). It is generally accepted that antigens must be highly purified prior to immunization and that antiserum must be of high titre, and low cross-reactivity for successful immunohistochemistry (194, 203). Antibodies are therefore'screened' prior to use in immunohistochemistry.

The choice of 'screening method' is dependent upon antibody. Optimal concentrations of multi-valent antigens mixed with bivalent antibodies in solution will combine to form three dimensional lattices which can aggregate and precipitate (193). An agar or agarose gel is commonly used to visualize these precipitates from small amounts of reagents placed in wells in the gel. The technique is known as immunodiffusion. Increased resolution of immunodiffusion may be made by applying a potential difference to separate antigen on the basis of charge; this technique is known as immunoelectrophoresis. Other effects e.g. agglutination and anaphylaxis which may occur after the primary reaction between antibody and antigen, may be used.

A technique that will be discussed in some detail is radioimmunoassay (RIA) a highly sensitive technique which can be employed for

'screening' antibodies. The basis of RIA is a high specific activity radiolabelled antigen and a specific antibody. This technique is employed for antigen molecules which do not precipitate with their specific antibody. Small molecules which may only prove antigenic as 'haptens' when coupled with high molecular weight 'carriers' will give rise to antibodies directed to themselves and to the carrier. If the antigen can be radiolabelled, the characteristics of the hapten antibody can be determined by RIA without interference from 'carrier' antibody molecules (212).

Radioimmunoassay was originally developed in 1960 by Yalow and Berson (213) for the measurement of peptide hormones; previous bio-assay methods had proved both inaccurate and insensitive. High specific activity radiolabelled antigen competes with unlabelled antigen for binding sites on specific antibody. Free and bound antigen may be separated by a variety of techniques e.g. paper electrophoresis, precipitation with a second antibody directed against the first antibody, precipitation with ammonium sulphate, or adsorption with talc or charcoal.

The titre of a number of antisera may be easily compared by determining the antiserum dilution required to bind, for example, 50% of labelled antigen. An antiserum of high titre will bind 50% of labelled antigen in high dilution, in contrast to an antiserum of low titre. The sensitivity of different antisera for measuring unlabelled antigen may be compared giving an indication of the avidity of interaction between the antisera and antigen. This forms the basis of the RIA standard curve for measuring amounts

of substances with high sensitivity. Mathematical treatments of avidity will be considered in chapter III.

The specificity of an antiserum may be studied by determining whether molecules related to the antigen are able to compete with and to displace radiolabelled antigen, when added to the assay. For example, an antibody with high specificity for the antigen will require large amounts of related molecules to reduce binding of radiolabelled antigen. An antiserum found to cross-react with a known substance may have the interfering antibodies removed by solid-phase absorption (214).

The principles of this radioimmunological technique have been discussed in some detail to demonstrate how the characteristics of an antiserum may be examined prior to use in immunohistochemistry.

3.2.C. Quantification

Immunohistochemistry has been discussed up to this point as a technique for studying the location of substances within tissues. A logical extension is the quantification or semi-quantification of specific staining. The technique might be able to localize changes with drugs, for example, of the specific fluorescence of a molecule, within a specific compartment of the cell, its intensity in other parts of the cell remaining unaltered. An important specificity control might correlate changes in the intensity of specific staining with biochemical tissue levels. It is important to note for immunohistochemical localization of enzymes, that increased fluorescence represents increased numbers of molecules rather than increased activity of the enzymes.

At a semi-quantitative level, photography of tissue sections from 'control' and 'stimulated' tissues with the same time exposure, have been used to detect differences in intensity,(e.g. 215). Optimizing the differences required appropriate diluting of the immunological reagents. At the quantitative level, care must be taken regarding variables such as section thickness and fading of immunofluorescence on exposure to ultra-violet light. Complex microspectrophotometric equipment has been used for quantification of immunofluorescence in tissue sections and recent work has used as a model, antigen covalently coupled to Sepharose beads for quantification (216).

The high sensitivity of 'PAP' immunohistochemistry, often equivalent to or greater than RIA (217), raises the interesting possibility that antigen may be localized in tissue sections even when it cannot be measured. Immunohistochemistry can reveal a high concentration of antigen in a very small and restricted population of cells, amongst a vast majority which do not possess the material. It is equally possible that immunohistochemistry could detect a change in localization of antigen, without a change in total tissue levels.

Immunohistochemistry has also proved to be useful in determining how an antigen is linked to its receptor site in the tissue. Sternberger (218) has shown that pre-treatment of pituitary tissue sections with LH-RH increases specific immuno-staining for this peptide. In this instance, immunohistochemistry is localizing increased amounts of exogenous LH-RH binding to active receptor sites in the tissue.

3.3. Application of histochemical techniques to the study of cyclic nucleotide systems.

The preceding sections of this introduction have demonstrated the importance, and the diversity of actions, of cyclic AMP and cyclic GMP in cell function. Whilst biochemical measurements give us precise information on the amount of cyclic nucleotides in a group of cells, they cannot tell us which cell types contain the nucleotides in a heterogenous tissue, and diffusion and degradation of cyclic nucleotides would also be expected to limit the value of sub-cellular studies. Micro-dissection, coupled with sensitive biochemical assay is a step closer if individual cell types can be dissected out, but the resolution of the technique is clearly limited, and information on localization in sites within cells cannot be obtained. An alternative approach of cells in culture, may help us to localize the nucleotides to neurones, glia or vascular cell types, but extrapolation 'in vivo' must be treated with caution.

Prior to the advent of cyclic nucleotide immunohistochemistry therefore, no information was available on the cell types in a tissue containing the nucleotides, or the distribution within these cells, factors essential to the understanding of cyclic AMP and cyclic GMP function.

Indirect evidence for the localization of cyclic AMP and cyclic GMP has come from attempts to localize components of the 'enzyme machinery' by conventional histochemical techniques, as described in the following section.

3.3.A. Conventional histochemical techniques.

Adenylate cyclase was localized by Reik et al in liver in 1970 (219), and was followed by reports of localization of catecholamine-sensitive adenylate cyclase in isolated capillary endothelium and/

pancreatic islets of Langerhans (220, 221).

These histochemical reactions used lead as a 'trapping' agent to visualize inorganic phosphate released from the cleavage of ATP by adenylate cyclase. A more specific substrate for adenylate cyclase has recently been used, which closely resembles ATP (5' - adenyl imidodiphosphate, AMP-PNP). Severe criticism was levelled at the use of lead in these reactions by Lemay and Jarrett (222) who showed that the concentrations of lead used could actually inhibit adenylate cyclase. The value of these reported localizations is clearly questionable therefore. Guanylate cyclase localization has also been similarly reported using GMP-PNP substrate (223, 224). Rechardt and Hörkönen (225) recently used cobalt as a 'trapping agent', and carefully controlled the histochemical reaction for localizing adenylate cyclase in C.N.S. tissue under the electron microscope. Reaction product was localized to the post-synaptic membrane; in the cerebral cortex, reaction product was localized in synapses using noradrenaline in the reaction medium, whilst in the striatum positive staining of synapses was observed with dopamine. Reaction product was also located in the plasma membrane of some nerve fibres, suggesting pre-synaptic as well as post-synaptic localization in the C.N.S. Alloxan, a specific inhibitor of adenylate cyclase, inhibited the reaction and haloperidol, a dopaminergic antagonist reduced, but did not eliminate specific staining in the striatum. These results clearly contrast with the astroglial and capillary distribution of adenylate cyclase found in the cerebral cortex by Joo et al using lead as the phosphate 'trapping' agent (226).

A post-synaptic localization of cyclic nucleotide phosphodiesterase in rat cerebral cortex was reported by Florendo et al (227) using an electron microscopic histochemical technique in 1971. 5'AMP formed from the breakdown of cyclic AMP with phosphodiesterase, was converted to adenosine and inorganic phosphate using purified 5' nucleotidase from snake venom; inorganic phosphate was trapped with lead. Lead was found to inactivate phosphodiesterase in homogenates, but was not found to inhibit phosphodiesterase after the gluteraldehyde fixation step used in the technique. However, a serious disadvantage of this technique was that gluteraldehyde fixation itself, was found to destroy approximately $\frac{2}{3}$ of the phosphodiesterase activity as measured biochemically. An approach to the question of fixation artefact was made in a study which showed the histochemical localization of phosphodiesterase in mouse cerebral cortex during development. Similar post-synaptic localization was found in fixed and unfixed tissue, although tissue preservation in unfixed tissue was naturally poor. This study showed localization of reaction product at developing synapses during the first post-natal month (228).

Recently, both cyclic AMP and cyclic GMP phosphodiesterase have been localized in rat brain, confirming post-synaptic localization (248). The technique was modified from the original procedure of Florendo et al in using crude snake venom rather than purified 5' nucleotidase. An interesting observation was that whilst cyclic GMP phosphodiesterase was clearly localized post-synaptically in cerebellum, cyclic AMP phosphodiesterase could not be histochemically detected in this brain area (229). Together with the possibility of phosphodiesterase loss from sections due to fixation and tissue buffer washing, a

further reservation concerns the high (3mM) substrate concentrations of the cyclic nucleotide required. The histochemical technique probably only localizes particulate, high Km phosphodiesterase. Another component of this cyclic nucleotide enzymatic machinery, phosphoprotein phosphatase, has been localized post-synaptically in rat cortex (229).

Conventional enzyme histochemical techniques have been of some use therefore in localizing components of the cyclic nucleotide system. Their specificity due to lack of 100% specific substrates has caused complications, and prevented localization of enzymes such as the cyclic nucleotide-dependent protein kinases. The close similarity of cyclic AMP and cyclic GMP not only to each other but also to a large number of structurally related nucleotides, clearly prevented the localization of these small molecules by conventional histochemistry.

3.3.B. Cyclic nucleotide immunohistochemistry

In 1969, Steiner et al (230) developed specific and sensitive antisera to cyclic AMP, and subsequently cyclic GMP, for use in radioimmunoassay (102, 103, 231). These antibodies allowed measurement of not only tissue levels of these nucleotides, but also, indirectly, measurement of adenylate cyclase and guanylate cyclase. To develop antibodies to these low molecular weight molecules, Steiner had postulated that in coupling them as 'haptens' to high molecular weight carriers in order to function as immunogens, the 3'5' phosphodiesterase moiety, and the groups on the purine nucleus, would have to remain intact. Consequently, cyclic nucleotides were substituted with a succinyl group at the 2'O position on the

ribose sugar, and conjugated to the 'carrier' at this position. The hapten-protein conjugates produced a reasonable titre of high specificity antibodies, when used to immunize rabbits and goats. Using antigen or succinylated antigen, radiolabelled with tritium or iodine, a radioimmunassay was developed enabling cyclic AMP or cyclic GMP to be measured in the range of 10^{-8}M to 10^{-9}M in tissue extracts, without prior purification. Previous techniques for measuring cyclic nucleotides using biological and chemical methods required the separation of cyclic nucleotides from other tissue nucleotides (232). The number of steps involved in these procedures precluded rapid analysis of large numbers of tissue samples, and generally the amount of tissue required was rather large due to limited sensitivity. The low cross-reactivity of cyclic nucleotide antibodies to ATP was especially important since this nucleotide is found in very high concentrations in the cell (231).

The development of a specific and sensitive radioimmunoassay to the cyclic nucleotides 'triggered' the expansion of the whole field of cyclic nucleotide research. Studies on cyclic GMP were perhaps most greatly affected, since the advent of RIA allowed measurement of the low tissue levels of this nucleotide simply and specifically. Recently, Cailla et al(233,234) have exploited the fact that the antisera have higher avidity for the succinyl cyclic nucleotide rather than the natural nucleotide, by developing a radioimmunassay for cyclic AMP and cyclic GMP that succinylates tissue samples prior to RIA. This modification results in assay sensitivity of the femtomolar level.

In 1972, following the development of these specific cyclic nucleotide antisera, three laboratories in the United States of America collaborated in the development of an 'indirect' immunofluorescence technique, to specifically localize cyclic nucleotides within unfixed frozen tissue sections, containing heterogenous cell types (215).

The first report (235) showed the specific localization of cyclic AMP in cryostat sections of rat salivary gland. In saline-injected control animals, positive staining was minimal, but in animals treated with isoproterenol, a drug known to increase levels of cyclic AMP in tissues, a marked increase in cyclic AMP fluorescence was seen in the basal portion of the acinar cells and the basket cells beneath them. The acinar cells are believed to be the target cells for adrenergic stimulation. This increase in fluorescence, shown to be specific for cyclic AMP, was not seen in other cell types. The following points summarize the salient features from this first paper employing cyclic nucleotide immunohistochemistry:

i) Localization of cyclic AMP to specific cell types in a tissue, and to a particular location within the cells, using a rabbit antibody to detect cyclic AMP in a rat tissue.

ii) Cyclic AMP must be 'bound' to receptor sites in some way and therefore localized; soluble or losely bound nucleotides would be washed away during the staining procedure.

iii) Biochemical increases in tissue levels of cyclic AMP correlate with increased staining of 'bound' cyclic AMP at specific sites.

The immunofluorescent localization of cyclic AMP in cerebellum

reported by Bloom et al (236) shortly after this report will be considered in detail, as the first application of the technique to the C.N.S. The biochemical and electrophysiological evidence for an involvement of cyclic AMP in the cerebellum has already been discussed.

Bloom et al localized this nucleotide to specific cell types in frozen sections taken from the rat cerebellum. When the cerebellum was frozen in isopentane pre-cooled with liquid nitrogen immediately after decapitation, positive staining was seen in the granule cell layer, the white matter, and 15-20% of the Purkinje cells; minimal fluorescence was observed in the molecular layer. Outside the cerebellar cortex, positive staining was seen in certain neurones of the cerebellum and underlying brain stem. The specificity of the cyclic AMP immunofluorescence was checked by the following criteria:

i) No staining was observed with the immunoglobulin fraction of control serum from unimmunized rabbits.

ii) Identical staining patterns were observed with antibodies from rabbits injected with hapten conjugated with either human serum albumin or keyhole-limpet hemocyanin carriers. This demonstrated that the specific staining pattern was not due to carrier antibody molecules, or cross-reacting protein antigens in the tissue.

iii) Specific staining was removed by prior incubation of antibody with cyclic AMP, but not related nucleotides.

When the time between decapitation and freezing was increased to 150 seconds, the number of Purkinje cells showing positive staining for cyclic AMP, rose to 80-90%, correlating with the post-mortem

decapitation increase in cyclic AMP. There was no change in the other cell types which showed positive staining for cyclic AMP.

In order to examine the neurotransmitter mechanism responsible for the increase in the number of Purkinje cells fluorescing, Siggins et al (237) applied solutions of neurotransmitters in Krebs-Ringer solution to the exposed surface of the cerebellar cortex. Immunofluorescence of sections from cerebella frozen after each treatment, were semi-quantified, by recording the number of Purkinje cells fluorescing in a number of folia in the cerebellar cortex, and calculating the mean percentage responding. Noradrenaline at 10^{-5}M caused 65% of Purkinje cells to fluoresce, in contrast to 15-20% response using control Krebs-Ringer or any other of the putative neurotransmitters tested. 10^{-3}M Noradrenaline caused approximately 90% of Purkinje cells to respond. In additional experiments, Siggins' group showed that electrical stimulation of locus coereleus, which innervates Purkinje cells in the cerebellar cortex via a noradrenergic pathway, increased the percentage of Purkinje cells fluorescing from 15-74%. This increase was not observed with sham stimulation of normal rats or after catecholamine-containing nerves had been selectively destroyed by prior treatment with 6-hydroxydopamine. In all these studies, changes in fluorescence were only observed in Purkinje cells and not in any of the other cell types showing cylic AMP fluorescence under basal conditions.

These immunofluorescence studies support the biochemical and electrophysiological studies suggesting that post-synaptic cyclic

AMP mediates noradrenergic transmission in the locus coereleus-Purkinje cell pathway. Three interesting points are raised by the immunofluorescent data:

i) What is the function of cyclic AMP found in the cell types, besides Purkinje cells, which did not respond to stimulation?

ii) Glial cells did not show positive staining for cyclic AMP in cerebellum, whilst tissue culture suggests both a neuronal and a glial pool for cyclic AMP (see section 1.4.).

iii) The immunofluorescent distribution of cyclic AMP is at variance with the distribution obtained by Rubin and Ferrendelli (169, 238), employing micro-dissection coupled with radioimmunoassay. These workers show that in freeze-dried cerebellar folia of mice, 43% of cyclic AMP is found in the molecular layer, 45% in the granular layer and 12% in the white matter. Cyclic AMP immunofluorescence shows very little staining in the molecular or white matter, the majority occuring in the granular layer and Purkinje cell bodies.

It seems apparent, therefore, that immunofluorescence may not localize all the cyclic AMP. Perhaps the fixation techniques are not adequate for preventing losses of cyclic AMP from the tissue sections during processing, or immunofluorescence is not sensitive enough to localize total cyclic AMP.

Immunofluorescent studies of cyclic nucleotides in cerebellum were limited to cyclic AMP, but indications that cyclic GMP might also be studied in the C.N.S, came from the work of Kebabian et al (239), who demonstrated that specific neurotransmitters elevate cyclic AMP and cyclic GMP in a peripheral nervous tissue, as demonstrated

by immunofluorescence. Using incubated slices of bovine superior cervical ganglion, from which frozen sections were subsequently cut and processed for cyclic nucleotide immunohistochemistry, dopamine increased cyclic AMP fluorescence predominantly in post-ganglionic neurones; no change in cyclic GMP fluorescence was observed. Acetyl choline was found to increase cyclic GMP fluorescence in axons and cell bodies of the post-ganglionic neurones, without affecting cyclic AMP fluorescence. The changes in cyclic nucleotide immunofluorescence with either acetyl choline or dopamine, in the presence of a phosphodiesterase inhibitor, correlated with increased tissue levels of the respective nucleotide, as measured by radioimmunoassay. In this study immunofluorescence demonstrated that specific increases in either cyclic AMP or cyclic GMP could occur in the same cell type, depending upon the neurotransmitter used for stimulation. Cyclic AMP/cyclic GMP changes at the same site clearly supported as 'Yin-Yang' control mechanism, possibly mediating post-synaptic potentials.

These examples cited, together with others which will be discussed in the text, show the successful use of cyclic nucleotide immunohistochemistry in localizing, and semi-quantifying, changes in cyclic nucleotides to specific cell types and loci within cells, amongst tissues with heterogenous cell types. Cyclic AMP is generally found cytoplasmically, but plasma membrane and nuclear membrane staining is found - in some tissues there is intranuclear fluorescence. Cyclic GMP is generally found in plasma membrane or nuclear elements, but cytoplasmic staining is also seen in certain cells (215).

It is perhaps remarkable that the technique is indeed successful. Cyclic nucleotides might be expected to be lost completely during buffer washes in the tissue processing, preventing any localization at all. Since cyclic nucleotides are localized, we are probably visualizing cyclic nucleotides at their site of action - bound to tissue receptors. The increases in fluorescence that have been observed in a number of tissues after different treatments, might suggest that a 'bonus' of the technique is that it is localizing 'physiologically important' nucleotides.

Cyclic nucleotide immunohistochemistry has also yielded information on the way in which cyclic AMP and cyclic GMP are bound to tissue sections (215). Cyclic GMP immunofluorescence in rat liver is substantially reduced at certain intracellular sites by exposure of tissue sections to 10^{-4}M EGTA or EDTA in buffer for 30 minutes at 37°C, prior to application of specific antibody. Staining of the nuclear membrane is not reduced, but fluorescence is lost in intranuclear elements and is reduced on the plasma membrane. This effect is blocked by addition of equi-molar concentrations of calcium chloride, whereas calcium chloride alone increases fluorescence at these sites. In sections from rat renal cortex from animals pre-treated with parathyroid hormone, a similar effect has been observed. In sections from saline-injected rats, this was not found (240).

These observations give us information on the importance of calcium ions for linking cyclic GMP at certain sites in the tissue. Whilst EGTA, EDTA and calcium chloride have no effect on cyclic AMP immunofluorescence in rat liver, cyclic AMP staining is markedly

diminished by exposure of the tissue to phosphate-buffered saline for 30 minutes at 37°C (215). This effect is prevented by reducing the sodium chloride concentrations of the buffer from 0.15 to 0.015M, or by adding Mg - ATP. These observations may be of interest in the binding of cyclic AMP to receptor proteins, since Corbin et al (241, 242) have shown that the activity of one form of cyclic AMP-dependent protein kinase is activated in a number of tissues by the high concentration of sodium chloride used in these experiments; additionally, the effect of saline on the kinase 'in vitro' is reduced by Mg-ATP.

A further point of interest regarding the binding of cyclic nucleotides to receptor sites in tissues, comes from the observations of Steiner et al (215) that in liver, different antibodies to cyclic GMP, although highly specific by RIA, may show slightly different abilities to localize different positive staining structures in tissues. This may be due to the heterogenous antibody population recognizing different structures with different avidity, perhaps due to stereospecific differences between the binding of cyclic GMP at different sites. Photo-affinity labels for cyclic GMP/cyclic AMP kinase should also be of use histologically for localizing receptor sites (243, 244).

Summarizing, cyclic nucleotide immunohistochemistry has yielded information not only on the distribution of cyclic nucleotides, but also on some of their characteristics to specific receptor sites. Whilst semi-quantitive increases in cyclic nucleotide immunofluorescence often correlate well with increases in tissue levels measured biochemically, there are a number of examples where

fluorescence intensity and distribution may vary, without net change in tissue levels, e.g. changes in distribution of cyclic GMP immunofluorescence during the course of liver regeneration, with no change in tissue levels of cyclic GMP (245). An added advantage of this technique, therefore, may be in detecting translocation of cyclic nucleotides and increases in bound:free nucleotide, sufficient to elicit a physiological response, without change in tissue levels of the cyclic nucleotide(246). A logical extension of the technique pioneered by Steiner et al, is its application for ultrastructural localization of cyclic AMP and cyclic GMP. This is currently being attempted by Steiner's group using 'PAP' techniques, and should prove to be a great use for understanding cyclic nucleotide function at different sites in the cell.

The most recent advance in cyclic nucleotide immunohistochemistry, however, has been the development of antisera to the receptor and catalytic sub-units of cyclic AMP and cyclic GMP-dependent protein kinases. These antisera are currently being used by Steiner's group for immunofluorescent localization in various tissues (247). In regenerating rat liver for example, translocation of cyclic AMP receptor sub-units from cytoplasm to nucleus has been detected, confirming biochemical data. Bloom et al (34) have very recently used antisera to the synaptic phosphorylated protein, protein I, to show a neuronal distribution using immunohistochemistry.

Clearly the use of immunohistochemistry for studying cyclic nucleotide systems, and interaction with other systems (e.g. using antisera to calcium-dependent regulating protein, CDR) is essential for understanding the roles of cyclic AMP and cyclic GMP as cellular regulators.

In the following chapters, experiments will be described which have attempted to confirm and extend the immunofluorescent localization of cyclic AMP in the C.N.S, and to develop a similar technique to localize cyclic GMP under a variety of 'in vivo' and 'in vitro' experimental conditions. Preliminary studies with antibodies to the cyclic nucleotide receptor proteins will be described in the final chapters,to demonstrate the potential of this approach for studying cyclic nucleotide systems in the C.N.S.

CHAPTER II

IMMUNOFLUORESCENCE METHODOLOGY AND NON-SPECIFICITY STUDIES

CHAPTER II

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1. IMMUNOFLUORESCENCE METHODOLOGY.

The indirect immunofluorescence technique adopted by Wedner et al (235) for cyclic nucleotide immunohistochemistry was employed, with certain modifications, throughout these studies.

1.1. Tissue preparation

Rat cerebellum was used in all experiments unless stated otherwise. Male, adult, albino Wistar rats weighing approximately 200g were obtained from the Bush Estate Animal Breeding Station, Penicuik, Scotland. Rats were housed not more than four to a cage, with food pellets and water available (ad libitum) and under normal lighting conditions, in the Pharmacology Animal House, University of Edinburgh. On transfer to the laboratory a period of at least 15 minutes was allowed prior to sacrifice, to enable the rats to settle down. Animals were killed by being stunned with a sharp blow, followed by decapitation with shears. The top of the skull and surrounding areas of bone were carefully removed to expose the cerebellum. This was then removed together with part of the underlying brain stem, and placed on a metal frame support which was plunged into a freezing mixture of isopentane (2-methyl butane) pre-cooled with liquid nitrogen (194). The time between decapitation and freezing was 2 minutes unless stated otherwise. For preparation of the freezing mixture approximately 200 ml of isopentane were placed in a Pyrex glass U-tube clamped and suspended in a Dewar flask (British Oxygen Co.) containing the liquid nitrogen. The tube was suspended until the contents formed a white crust at the bottom. At this time the tube was removed from the liquid nitrogen allowing the crust to thaw, and the metal tissue holder was immersed. After freezing for 1 minute, the holder was removed and the tissue rapidly transferred to a cryostat (Linde, West Germany)

situated close by, having an interior temperature of -25°C; the tissue was allowed to equilibrate inside the cryostat for at least 30 minutes. After this period of time, the tissue was fixed onto a small cork mat with a drop of water, and allowed to freeze inside the cryostat. The mat, with the attached tissue, was then affixed to a cryostat chuck in a similar manner. The chuck was subsequently assembled in the cryostat and 6-10 μm coronal frozen sections were cut.

A number of factors determined the 'quality' of the tissue sections e.g. i) the position of the P.T.F.E. coated glass slide, which rested against the blade to prevent sections 'curling' during cutting ii) the angle which the cryostat knife was mounted at. The most important factor, however, was that the knife was very sharp and free from 'burrs' or scratches. A paint brush was used to remove damaged sections adhering to the knife during cutting. Selected sections were removed by positioning a gelatin/chrome-alum coated glass slide (see appendix 1) over the section; the temperature differential between the 'warm' slide (at room temperature) and 'cold' blade, caused the section to attach, and with experience, sections free from wrinkles could be obtained. 'Coating' prevented sections being lost from the slides during the tissue processing for immunofluorescence. Tissue sections were allowed to dry at room temperature for approximately 30 minutes, prior to processing for immunofluorescence. After cutting, tissue blocks were either discarded or could be stored over ice in a small beaker sealed with 'cling-film' (C.E. Payne & Sons Ltd) in the cryostat, to prevent freeze-drying.

Note: For experiments referred to in Dr Steiner's laboratory in the University of North Carolina, male, adult Sprague-Dawley rats of approximately 200g (Charles River, U.S.A.) were employed, and sections were cut on a Damon/IEC cry ostat at 4 μm.

1.2. Immunofluorescence staining.

Experiments were performed on unfixed frozen tissue sections unless stated otherwise. Phosphate-buffered saline (PBS, see appendix 2) was used for all antibody dilutions and buffer washes.

Tissue sections were incubated for 30 minutes or overnight (15 hours) where stated, with 10-20 μl of diluted rabbit antibody (immunoglobulin fraction), which had been centrifuged for 3 minutes at 13,000g (Eppendorf centrifuge) to remove aggregates. Incubations were performed in glass Petri dishes (4" diameter) containing damp filter paper circles, to act as humid containers, preventing evaporation of antibody solutions. The dishes were rested on a flat surface and left undisturbed at room temperature for the duration of the incubation period. On completion of incubation, slides were carefully removed and blotted dry with paper tissues around the sections. A glass Pasteur pipette was used to carefully wash the antibody solutions away from the tissue sections with buffer. The slides were then placed in a stainless steel rack and completely immersed in a glass staining trough containing approximately 400ml of buffer. A maximum of 6 slides with 4 sections on each were processed in each experiment, sections being positioned such that adjacent antibody solutions would not 'run' and contaminate each other during the incubation. It was very important to ensure that the tissue sections were not allowed to dry-out at any stage during processing, since this resulted in fluorescent

artefacts due to concentration of buffer salts on the tissue. The staining rack was gently agitated during the buffer washes, and after 3 minutes was transferred to a similar staining trough containing fresh buffer; the slides received a total of 3 x 3 minute washes.

Sections were then incubated at room temperature for a further 30 minute period with a 1:10 dilution of FITC swine anti-rabbit serum IgG (Dakopatts, Denmark. Supplied by Mercia Diagnostics, Watford, England)centrifuged prior to use as described previously. The fluorescent 'second antibody' was used to detect sites of IgG binding by the 'first' rabbit antibody. The buffer washings were repeated as before. On completion, slides were removed, blotted dry around the sections and covered with a drop of mountant, 50% glycerol in PBS, followed by careful positioning of a clean glass cover slip (Chance-Proper Ltd, England). Excess mountant was removed,and slides were viewed under ultra-violet light using a Carl Zeiss 'large fluorescent microscope' with type III FL vertical illuminator, which incorporated transmitted and reflected light (Episcopic) illumination systems. For low magnification microscopy using transmitted light, a x10 FL-NEOFLUAR objective of numerical aperture (N.A.) 0.3 was used, whilst for higher magnification a x40 Ph2 FL-NEOFLUAR objective of N.A. 0.75 was employed,with reflected light illumination. Additional microscope magnification was provided using a x8 ocular and x1.25 Zeiss Optovar. Illumination was provided by means of an HBO 200 'super pressure' mercury lamp (Osram, Germany). The filter system employed BG3 primary and 50 Schott secondary filters together with FL 500 reflector. A sub-stage condensor of N.A. 1.4 yielded a dark background for transmitted light fluorescence microscopy.

The power source was switched on approximately 30 minutes prior to use, to stabilize fluorescence intensity. During observation it was important to minimize 'fading' of the fluorochrome, by preventing exposure of one area of fluorescence on the tissue for an extended period of time.

An adjustable 'beam splitter' on the microscope enabled 100% of visible light to reach the ocular during observation, and 100% of light to reach an Olympus OM-1 35mm camera for immunofluorescence photomicrography. Sections were photographed using 1 minute exposure time on Ilford HP5 high-speed black and white film (A.S.A 400). Exposed films were developed with Ilford Microphen developer according to the manufacturers instructions. Negatives were enlarged and printed on Ilford Ilfospeed grade 5 high-contrast photographic paper. Sections were usually photographed directly after immunofluorescent processing, but slides could be stored at $4^{o}C$ in a 'light-tight' box for 2 - 3 days without causing significant fluorescence fading.

Also mounted in the microscope was a 120W white light source enabling sections stained by conventional toluidine blue histology to be photographed; exposure times were selected between $^{1}/_{15}$ and $^{1}/_{60}$ second.

For calibration of photomicrographs, a graticule engraved at $10/^{\mu}m$ intervals on a microscope slide was photographed using the different objectives, and the negatives were printed identically to those of the experimental sections.

Note: For experiments referred to in Dr Steiner's laboratory there were two modifications to the previously described methodology:

i) the fluorescent antibody employed was supplied by Miles Laboratories,

Indiana, U.S.A.:- FITC goat anti-rabbit serum IgG, code number 65-173, which was employed at a 1:50 dilution. ii) A Leitz Orthoplan fluorescent microscope with 480-510μm filters and x10, x40 and x63 (OIL) fluorescent objectives was used, coupled with a Leitz Orthomat automatic camera containing Kodak Tri-X black and white film (A.S.A. 400).

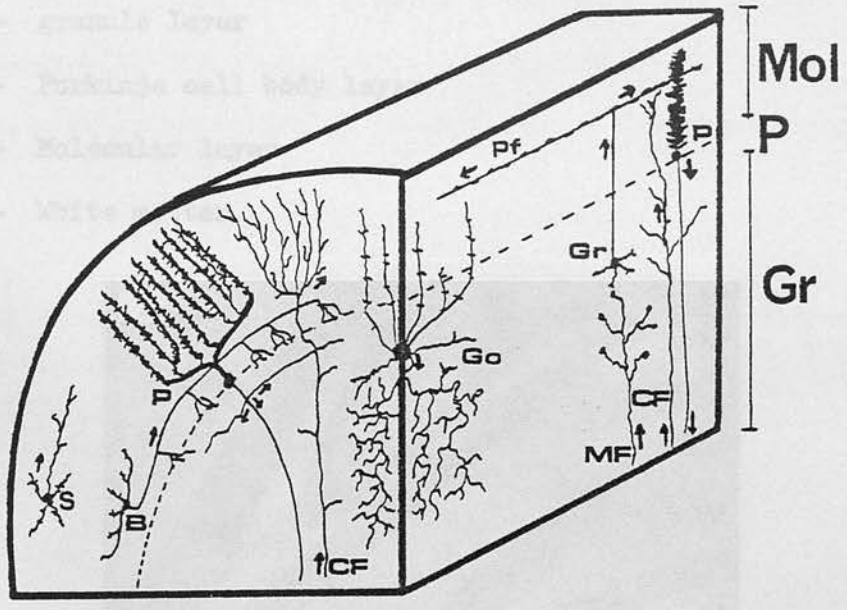
1.3. Preliminary attempts to localize cyclic AMP in rat cerebellum

The neuronal components and histological structure of the mammalian cerebellum by toluidine blue staining (see appendix 4), are shown in figures 8 and 9.

Preliminary experiments on cyclic nucleotide immunofluorescence were carried out using the immunoglobulin fraction of a number of rabbit antibodies to cyclic AMP and cyclic GMP prepared in the M.R.C. Brain Metabolism Unit by Mr T Attree prior to the start of my work. Surprisingly, similar staining patterns were found not only between the cyclic nucleotide antibodies, but also with rabbit non-immune immunoglobulins. The staining in the granule and Purkinje cell body layers of the cerebellar cortex was very similar to that reported for the specific localization of cyclic AMP in rat cerebellum (236, 237), and raised considerable doubts as to the specificity of the technique.

The following section considers the possible causes of the non-specific immunofluorescence which was encountered.

Figure 8 Neuronal components of adult mammalian cerebellum



Redrawn from (249)

Cell layers are shown on the right side of the diagram:

Mol - Molecular layer

P - Purkinje cell body layer

Gr - Granule layer

(White matter lies below the granule layer)

Inputs: Mossy fibre (MF) and climbing fibre (CF)

Principal neurone: Purkinje cell (P)

Intrinsic neurones: Granule cell (Gr), stellate cell (S), basket cell (B) and Golgi cell (Go)

Parallel fibre (pf)

Figure 9 Histological cell layers of adult rat cerebellum stained using toluidine blue.

a) Low magnification. Calibration bar = 500 μm

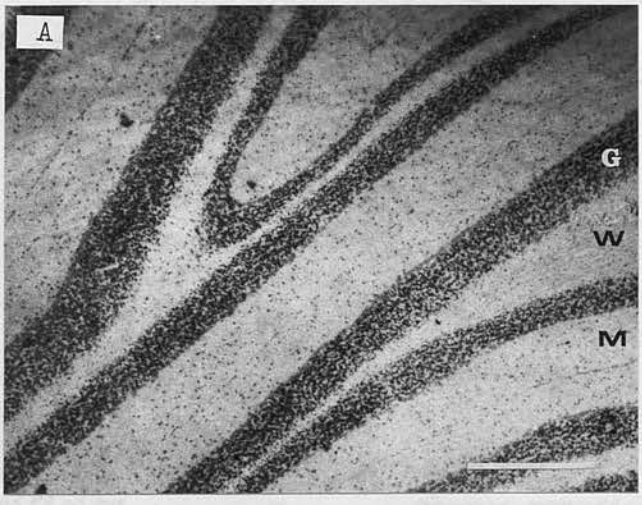
b) High magnification. Calibration bar = 100 μm

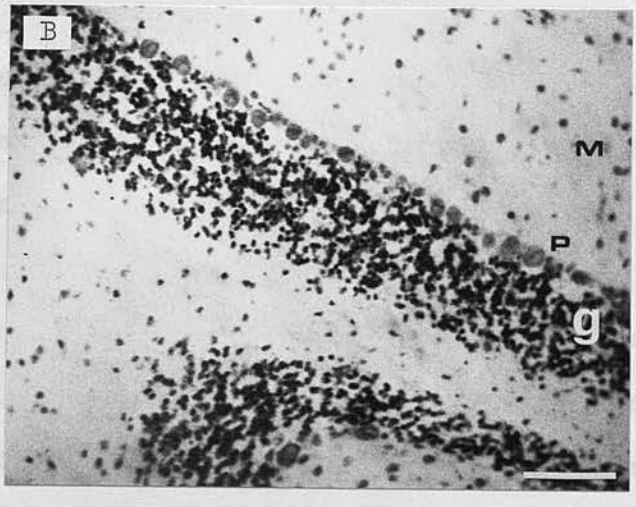
g/G - granule layer

P - Purkinje cell body layer

M - Molecular layer

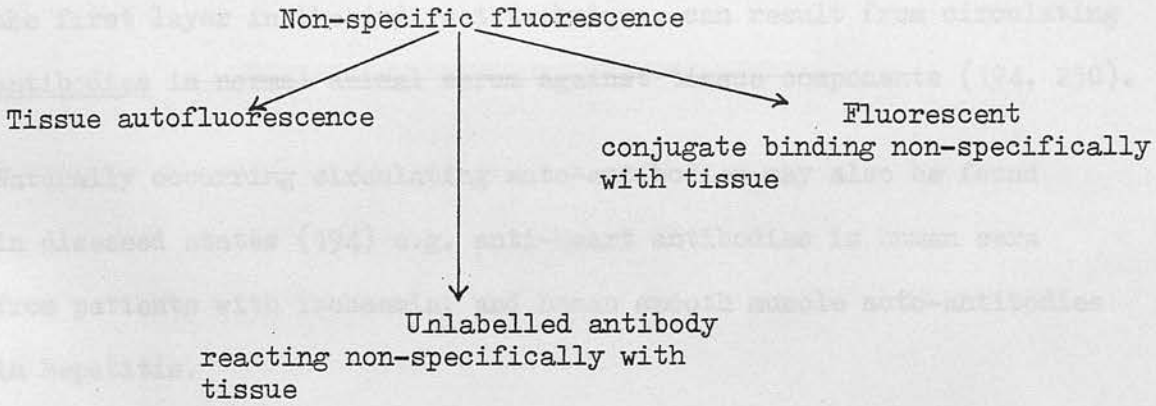
W - White matter





2. NON-SPECIFIC IMMUNOFLUORESCENCE

By definition, non-specific immunofluorescence is that which is not due to the specific interaction between antibody and antigen under investigation. The diagram below shows the possible causes of non-specific fluorescence in the 'indirect' technique, when non-immune serum/immunoglobulin is employed as the first layer.



a) Immunological non-specificity – contaminating antibodies

b) Non-immunological non-specificity – charge reaction between IgG and tissue

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To determine which form of non-specificity was responsible for the staining observed in cerebellar sections, buffer was applied instead of non-immune immunoglobulin as the first 'layer', followed by fluorescent conjugate. The absence of fluorescent staining indicated that the binding of the conjugate was not the cause of the non-specificity. Occasionally, however, small yellow-red autofluorescent granules were observed in the cytoplasm of Purkinje and other large cells, probably representing lipofuchsin, although the colour of fluorescence could be clearly distinguished from the apple-green FITC fluorescence. Non-specificity in cerebellar sections with non-immune immunoglobulin, however, was most clearly observed as staining of the

granule and Purkinje cell body layers, but was also found in the walls of large blood vessels, scattered throughout the white matter, and at the apex of ependymal cells of the choroid plexus and lining the ventricles (for example, see figures 10 and 37).

2.1. Immunological non-specificity

Non-specificity due to the unlabelled immunoglobulin fraction of the first layer in the indirect technique, can result from circulating antibodies in normal animal serum against tissue components (194, 250).

Naturally occurring circulating auto-antibodies may also be found in diseased states (194) e.g. anti-heart antibodies in human sera from patients with ischaemia, and human smooth muscle auto-antibodies in hepatitis.

2.2. Non-immunological non-specificity

Whilst a large amount of data exists on protein-protein reactions between fluorescent conjugates and tissues in direct immunofluorescence e.g. (251, 252, 253) few reports have studied non-specific reactions due to unlabelled antibody and tissue (254, 255). This may be because non-specific reactions only occur with high concentrations of primary antiserum and not with specific antiserum of high titre in the indirect technique (256), allowing dilution to remove non-specific staining, whilst retaining specific immunofluorescence.

A number of attempts to increase immunofluorescence staining intensity in the indirect technique by producing a concentrated immunoglobulin fraction of the unlabelled antiserum, have indicated that non-specific reactions are associated with this antibody fraction (255). In brain, for example, Hartman (257) has found that the use of IgG rather than

whole serum significantly increases non-specific background staining.

In 1964 Allerand and Yahr (258) demonstrated binding of the IgG fraction of normal human sera on ethanol-fixed cryostat sections of normal human and monkey nervous tissue, using indirect immunofluorescence. The reaction, which was not dependent upon complement, was not seen with other serum fractions, indicating a degree of specificity for the IgG binding.

IgG contains a heterogeneous population of molecules with respect to charge, as observed by the range of electrophoretic mobility of IgG (193). The different charges on the different molecules are indicative of variations in amino acid structure (259). At neutral pH however, IgG has a net negative charge.

Fractionation employing carboxymethyl-cellulose has been used to separate IgG into 'fast' γ_1 and 'slow' γ_2 fractions with respect to mobility (260); γ_1 is resistant to papain cleavage (see page 104) whilst γ_2 is sensitive. A non-specific reaction of the γ_1 fraction of human IgG with erythrocytes has been reported (261). The charge heterogeneity of IgG might suggest that one electrophoretic component could be responsible for non-specific binding of IgG in cerebellar tissue sections.

Immunofluorescent studies on skeletal muscle, to determine whether sera from myasthenia gravis patients have a muscle auto-antibody (262), showed non-specific reactions due to IgG in normal sera with similar staining patterns in normal and myasthenic sera. Aarli et al (263, 264) were able to show by repetitive tissue absorptions that the non-specific reactions were not due to a small part of the serum IgG

e.g. γ_1 or γ_2, but that most of the IgG had the capacity to bind to muscle tissue non-specifically. Similar results were found with not only human IgG but also rabbit IgG, suggesting that the non-specific reactions with muscle tissue were a general feature of IgG molecules.

2.2.A Experiments to determine whether non-specificity was mediated by IgG.

Experiments were performed using frozen sections of rat cerebellum.

i) Immunoglobulin fractions obtained from non-immune sera from a number of rabbits showed identical staining patterns at concentrations of approximately 1mg protein/ml, the intensity of non-specific staining increasing with immunoglobulin concentration.

ii) The non-specific binding of immunoglobulin was not dependent upon complement since no effect was found by heating rabbit immunoglobulin to 65°C for 30 minutes.

iii) A generalized form of protein-binding to the tissue section could not account for staining non-specificity since substitution of 10mg/ml of crystallized rabbit albumin (Sigma Chemical Co, Norbiton, Surrey) and a 1:10 dilution of FITC goat anti-rabbit albumin (Nordic Immunologicals, Maidenhead, Berkshire) as immuno-histochemical reagents, failed to show non-specific immunofluorescence.

iv) Purified rabbit IgG (Pentex, Miles Laboratories, Slough, Buckinghamshire, Code No. 64-147, Lot No. 47) showed clear non-specific staining at concentrations of approximately 0.5mg/ml, the intensity of non-specific staining increasing with IgG concentration.

v) To determine whether the binding of IgG to C.N.S. tissue was species-specific, cerebellar sections from either rats or mice (CFI strain) were stained with rabbit IgG as above, or human IgG (Scottish National Blood Transfusion Service) using an FITC swine

Figure 10 Non-specific immunofluorescence of unfixed rat cerebellar sections with non-immune rabbit and human immunoglobulin and IgG.

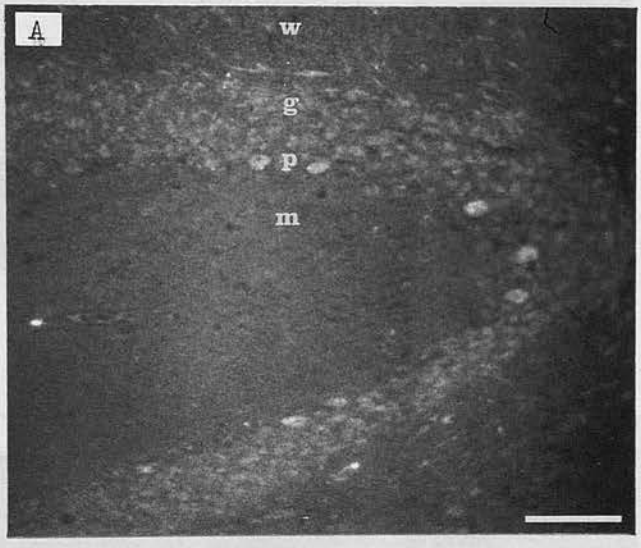
a) Normal rabbit immunoglobulin. Calibration bar = 100 μm

b) Normal rabbit immunoglobulin. High magn. Calibration bar = 50 μm

c) Normal rabbit IgG. Calibration bar = 100 μm

d) Normal human IgG. Calibration bar = 100 μm

g - granule layer
P - Purkinje cell body
m - molecular layer
w - white matter



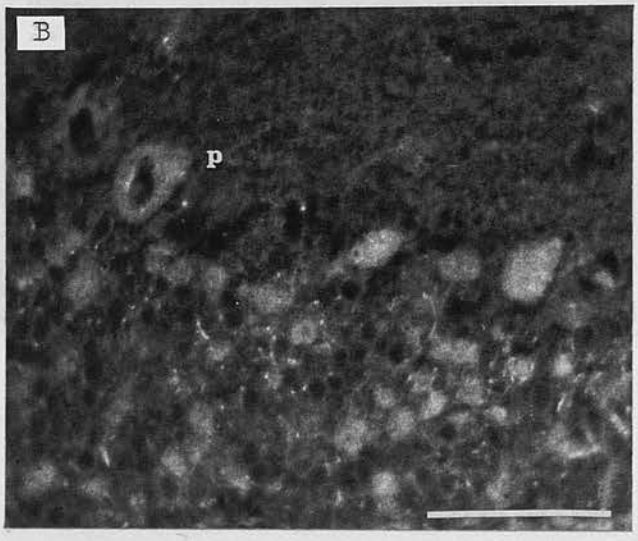
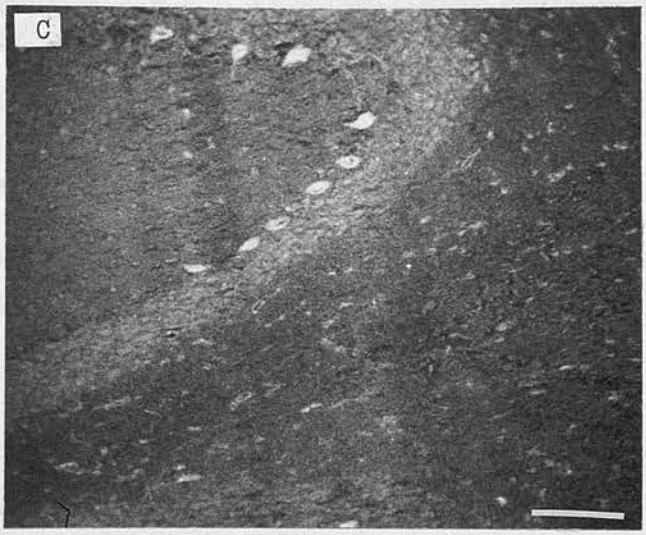
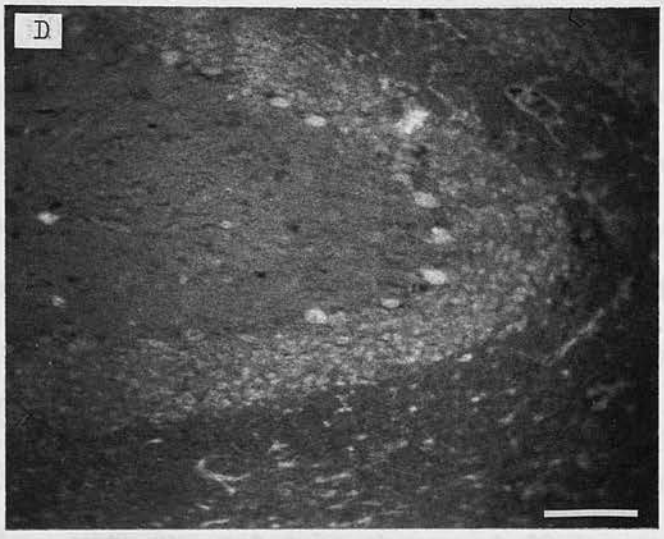


Figure 10 (Continued)





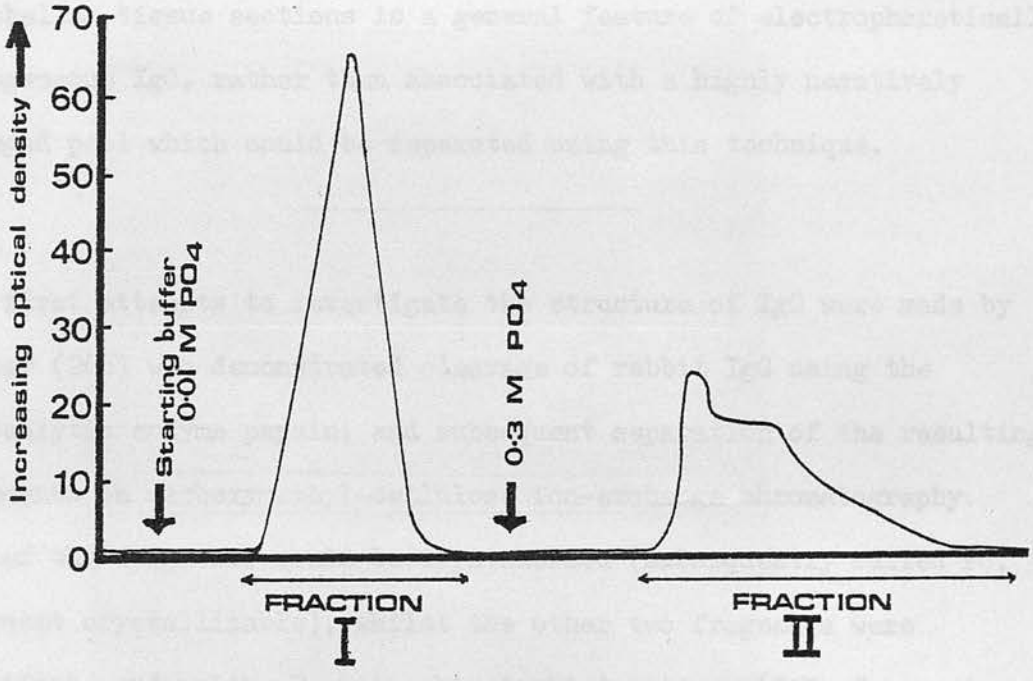
anti-human serum IgG conjugate (Nordic Immunologicals); similar results were obtained in all cases.

2.2.B. Experiments to determine whether a component of IgG responsible for non-specificity could be separated by ion-exchange chromatography.

Fractionation of rabbit IgG on DEAE-cellulose ion-exchange gel..

Diethylaminoethyl (DEAE)-cellulose (medium mesh, Sigma Chemical Co) was swollen and washed with distilled water in a beaker to remove 'fines', followed by extensive washing with 0.01M sodium-potassium phosphate buffer, pH7.4. A Pyrex 35x1cm^2 glass column was then filled with gel to a height of approximately 25cm, and run through several times with buffer from a reservoir, ensuring that the gel was packed well. The flow rate of the eluate was controlled by means of a clip on the plastic outlet tube. 2ml of buffer containing 30mg of immuno-electrophoretically pure rabbit IgG (Pentex, Miles Laboratories, Code No. 64-147, Lot No. 47) were then carefully layered onto the top of the column with a Pasteur pipette, and the buffer was passed through, ensuring that the level did not fall below the top of the gel. The optical density of the column eluate was recorded continuously at 280nm using an ultra-violet spectrophotometer coupled to a pen-recorder (LKB, Sweden) in order to monitor protein elution (see figure 11). Samples were collected using a fraction collector (LKB, Sweden) with pre-set tube change at 20 drops (approximately 1.5ml), peak I being collected between fractions 16 and 25. After baseline optical density level had been re-established, the molarity of the column buffer was changed to 0.3M sodium-potassium phosphate, pH 7.4. A broad peak was eluted from fraction 44 and was collected (peak II), its shape suggesting that it might be further resolved using a

Figure 11 Fractionation of rabbit IgG by DEAE-cellulose ion-exchange chromatography.



continuous gradient elution system (265). The eluted fractions, peaks I and II, were extensively dialyzed at 4°C against 5 litres of distilled water (see page 121) over an 18 hour period. The fractions were frozen at -25°C and freeze-dried (see also page 121).

To determine whether non-specific staining resided in peak I or peak II eluates, immunofluorescence was performed under identical conditions with 5mg protein/ml of each peak eluate. Strong staining was observed

with the peak I fraction, whilst peak II eluate staining was very weak (see figure 12).

Since peak I eluate contains a relatively homogeneous population of IgG molecules with respect to charge, whilst peak II eluted with high molarity buffer contains a more highly electronegative population (265), the results suggest that non-specific immunofluorescence in cerebellar tissue sections is a general feature of electrophoretically homogeneous IgG, rather than associated with a highly negatively charged pool which could be separated using this technique.

The first attempts to investigate the structure of IgG were made by Porter (266) who demonstrated cleavage of rabbit IgG using the proteolytic enzyme papain, and subsequent separation of the resulting fragments on carboxymethyl-cellulose ion-exchange chromatography. One of the fragments could be crystallized (subsequently called Fc, fragment crystallizable), whilst the other two fragments were identical, and unlike Fc were able to bind antigen (FAB, fragment antigen binding). Subsequent work by Nisonoff et al (267) showed that the enzyme pepsin, could cleave IgG to form an $(FAB)_2$ complex and further work by Porter and Edelman using reduction and alkylation, isolated the constituent 'heavy' and 'light' chains of IgG (268).

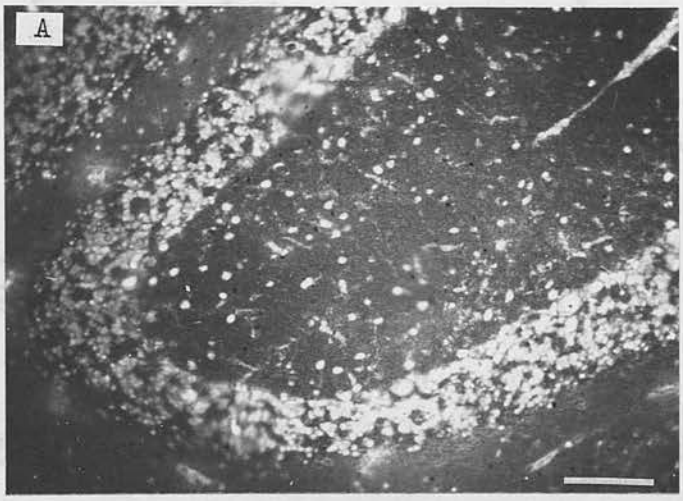
Porter's model of IgG (see figure 13) has been confirmed using electron microscopy of antigen-linked IgG molecules, which clearly show a flexible Y-shaped structure.

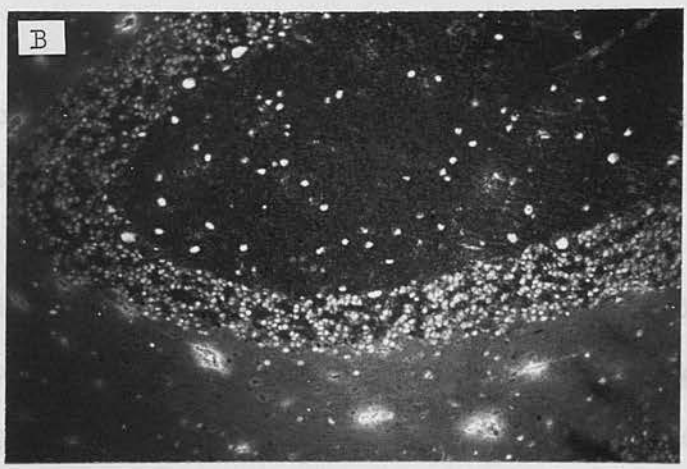
Figure 12 Non-specific immunofluorescence of rat cerebellar tissue sections with rabbit IgG and fractions obtained after DEAE-cellulose ion-exchange chromatography (sections lipid-extracted)

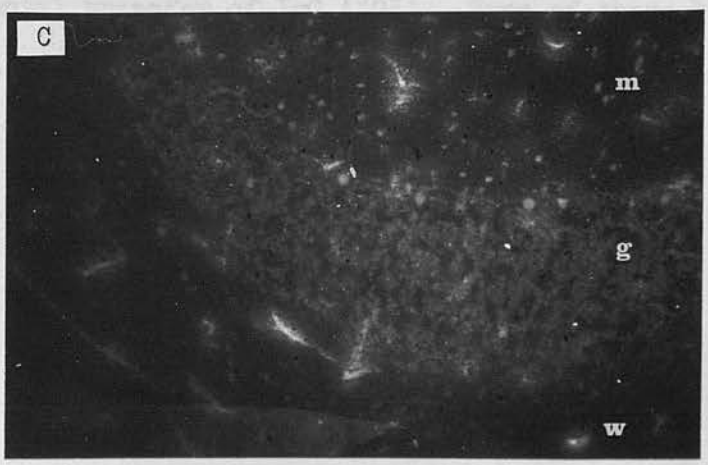
a) Rabbit IgG
b) DEAE-cellulose fraction I
c) DEAE-cellulose fraction II

Calibration bar = 100 µm

g - granule layer
m - molecular layer
w - white matter







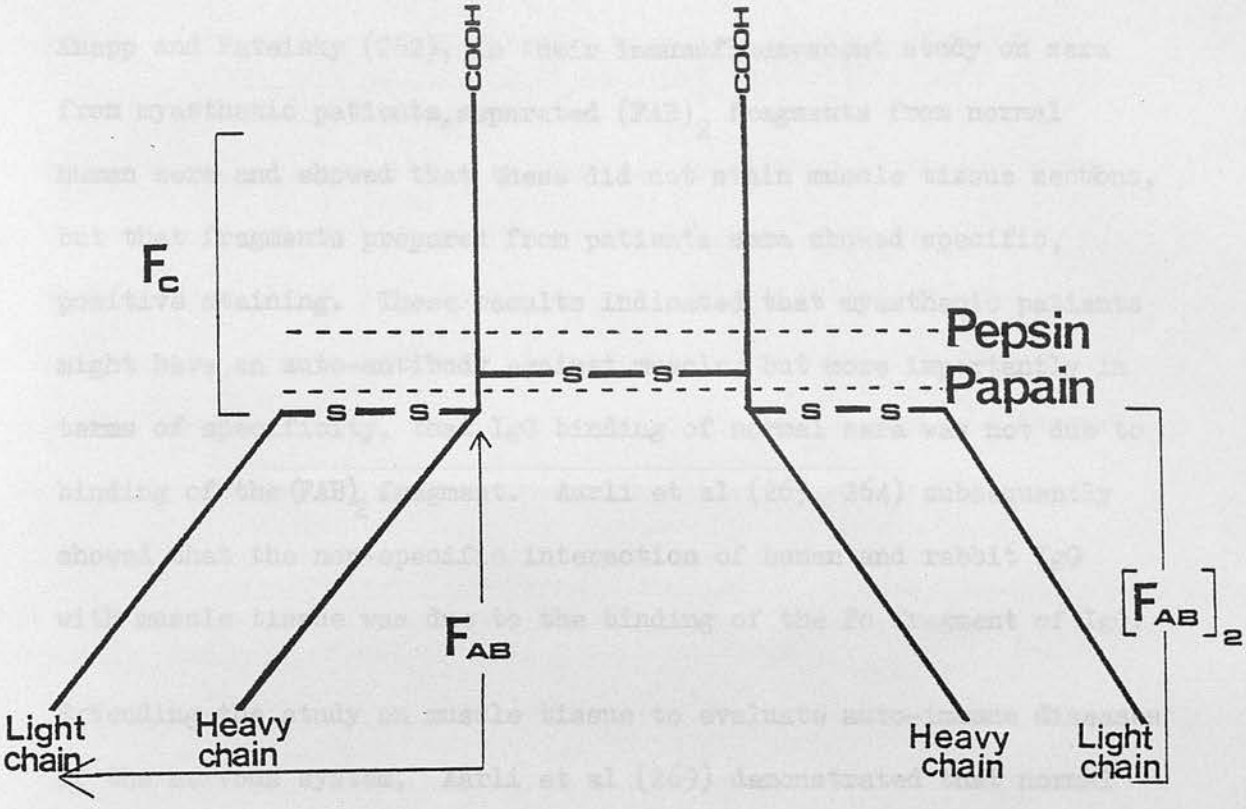


Figure 13 Porter's model of IgG showing sites of cleavage by pepsin and papain.

The FAB portion of IgG, responsible for antigen binding, exhibits specificity of immune reaction due to the linear and three-dimensional structure of amino acid sequences at the ends of the heavy and light chain regions of the FAB molecule. In contrast, the Fc portion of the molecule has been shown to be involved in the 'biological' properties of IgG e.g. binding to macrophages and mast cells, and regulation of the placental transfer of IgG (193). Some of these biological effects e.g. complement fixation, are only expressed after the antibody has bound antigen.

Knapp and Pateisky (262), in their immunofluorescent study on sera from myasthenic patients, separated $(FAB)_2$ fragments from normal human sera and showed that these did not stain muscle tissue sections, but that fragments prepared from patients sera showed specific, positive staining. These results indicated that myasthenic patients might have an auto-antibody against muscle, but more importantly in terms of specificity, that IgG binding of normal sera was not due to binding of the $(FAB)_2$ fragment. Aarli et al (263, 264) subsequently showed that the non-specific interaction of human and rabbit IgG with muscle tissue was due to the binding of the Fc fragment of IgG.

Extending the study on muscle tissue to evaluate auto-immune diseases of the nervous system, Aarli et al (269) demonstrated that normal human IgG binds to myelin sheafs, glia and neurones of the C.N.S. Immunofluorescence and tissue absorption studies demonstrated that the non-specific binding was due to interaction of the Fc region of IgG with C.N.S. tissue. Their results demonstrated that the method of tissue fixation influenced the staining pattern, and that maximal non-specific fluorescence was obtained after lipid extraction of the tissue sections with chloroform-methanol (2:1). The 'patchy' staining observed with unfixed tissue was interpreted as being due to the high lipid content of tissue sections from the C.N.S. The need for fixation to remove lipid, interfering with specific immunofluorescence in C.N.S. tissue sections, has been commented upon (270).

2.2.C. Experiments to examine the effect of lipid extraction of rat cerebellar tissue sections on non-specific immunofluorescence.

6μm frozen sections of rat cerebellum were immersed in ice-cold chloroform -methanol (2:1) for 30 minutes, followed by washing of the sections for 3 x 3 minutes in phosphate-buffered saline. Sections were then processed for immunofluorescence. Lipid extraction was found to eliminate the diffuse staining as observed in unfixed sections, whilst intensifying non-specific staining in other structures (see figures 10 and 14).

The Fc interaction with C.N.S. tissue sections reported by Aarli et al (269) was shown to be due to binding of the fragment with small tissue protein molecules of high basic charge. Less intense non-specific reactions were also observed in other tissues e.g. liver and kidney. Fc interaction has also been suggested as the reason for frequent non-specific staining of collagen due to binding of IgG to basic groups on the fibres (271).

In sections of rat cerebellum processed for immunofluorescence with Fc fragment from human non-immune IgG, Aarli et al (269) reported that staining of the granule layer was the most prominent feature, with Purkinje cells also being stained. These observations with Fc binding are clearly comparable with my observations using rabbit and human IgG.

It is important to point out that the'biological'effects of IgG mentioned previously, may be mediated via binding to specific Fc receptors e.g. in lymphoreticular tissue sections (272). These Fc receptors are assumed to be distinct from the binding sites contributing to non-specific immunofluorescent staining, since

Figure 14 Non-specific immunofluorescence of lipid-extracted rat cerebellar tissue sections with rabbit IgG.

a) Low magnification. Calibration bar = 100 μm

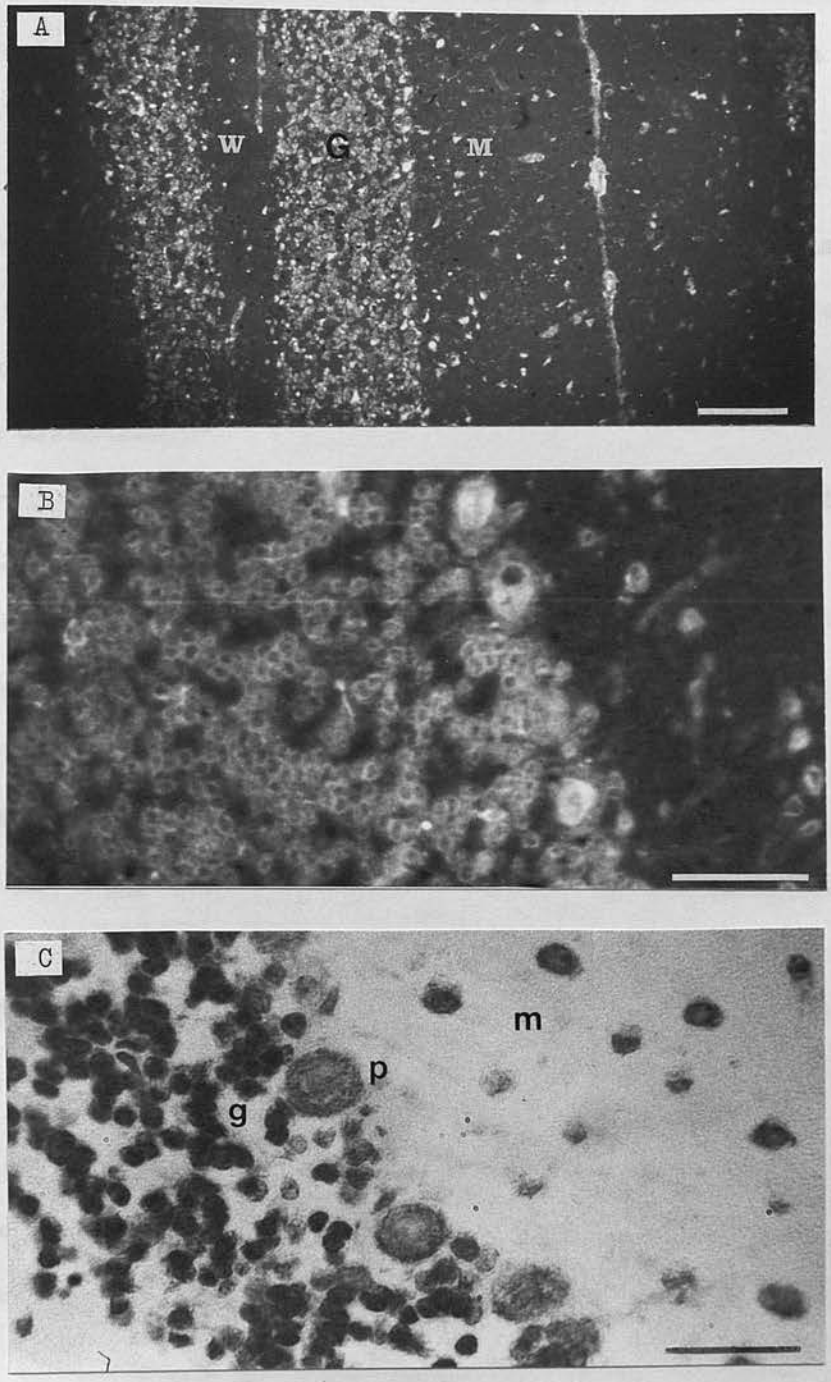
b) High magnification. Calibration bar = 50 μm

c) Toluidine blue histological staining for comparison with b) High magnification. Calibration bar = 50 μm

g/G - granule layer

m/M - molecular layer

P - Purkinje cell body



antigen-antibody complexes are required for Fc receptor binding, the antibodies often showing sub-class specificity (273).

Whilst non-specific staining due to the first antibody is rarely considered in standard texts, it is clear that many workers have experienced similar problems often vaguely described as resulting from 'protein-protein' interactions or contaminating antibodies, which are due to Fc reactions. An example is cited from a review of immunocytochemistry of GABA-ergic systems in the brain by Roberts (274):

"the blank reaction observed in control sections treated with non-immune serum must be reduced to an absolute minimum. Too early compromises with a high blank and the resultant attempts to interpret shades of differences between similarly stained sections can be disastrous."

If non-specific staining is clearly distinct in distribution from specific staining, separation may be a simple task. This point is stressed by Pearse (275) who states that if the specific distribution of a molecule e.g. using another technique, is known in advance, immunofluorescence can give precise and accurate results. In these studies reported here, however, non-specific staining patterns in cerebellum were similar to those reported for specific localization of cyclic AMP; difficulties in interpreting specific and non-specific staining using liver antigens has also been reported (255).

2.2.D. Attempts to remove non-specific staining.

i) Dilution of immune and non-immune immunoglobulin.

Parallel dilutions of immune and non-immune immunoglobulin fractions were made in an attempt to'dilute-out'weak non-specific staining

present at approximately 1mg protein/ml and above, whilst retaining specific staining. Removal of non-specific staining by dilution, was associated with removal of staining with immune sera, however, preventing any distinction. A variety of antibodies to cyclic AMP and cyclic GMP of differing titre were used unsuccessfully in an attempt to 'select' an antibody showing differences from non-immune immunoglobulin, at similar protein concentrations.

ii) Absorption of unlabelled antibody with tissue powders has been used by a number of workers with some success e.g. (274). Hartman (257) reported limited success in removing non-specific fluorescence with acetone powders, for localization of dopamine-β-hydroxylase in C.N.S. tissue.

Care should be taken in experiments using tissue powder absorptions to remove non-specific immunofluorescence, since the powder may remove normal IgG by Fc binding, instead of having the desired effect of removing 'anti-tissue' antibodies. A reduction in specific staining or background staining might therefore be observed, simply as a result of a decreased IgG concentration. To demonstrate this 500μl of non-immune immunoglobulin were incubated for 30 minutes at room temperature (with occasional shaking) with 100mg of PBS-washed liver powder (see appendix 6). After centrifugation at 13,000g for 3 minutes (Eppendorf cent. supernatant was removed and the procedure was repeated. 5μl of the centrifuged supernatant were then assayed for IgG using a Miles agarose radial immunodiffusion kit (Miles Laboratories, Slough, Buckinghamshire). A 46% reduction in IgG was found compared with un-incubated non-immune immunoglobulin. A further consideration in the use of tissue powders for removing 'anti-tissue' antibodies, is that the tissue antigen might be destroyed by the acetone used for preparation of the powder.

iii) In PAP immunohistochemistry, sections are first treated with serum from a different species to that in which the specific unlabelled antibody is raised, to 'block' background staining (276). Experiments carried out with concentrations of human IgG up to 15mg/ml to pre-treat sections for 30 minutes in the indirect technique, failed to prevent non-specific binding of rabbit immunoglobulin however. This was presumably due to interchange between human and rabbit IgG for non-specific binding sites.

iv) Oxidative deamination of cerebellar tissue sections was carried out with 2% nitrous acid (1 minute) followed by a short wash in 2% acetic acid and finally 3 x 3 minute washes in PBS, in an attempt to block the basic groups in C.N.S. tissue sections believed to be responsible for non-specificity (253). No reduction was found in non-specific immunofluorescence however.

v) An experiment was performed using 1% Triton X-100 (Sigma Chemical Co) incorporated in the antibody solutions and buffer washes. This compound, which is a non-ionic detergent, has been shown by Hartman (257) to reduce non-specific staining, presumably by interfering with the low avidity binding of non-immune immunoglobulin. No significant effect was found however.

vi) The possibility of increasing antibody pH to remove non-specificity by decreasing the charge interaction between antibody and basic tissue proteins (277) was not attempted on the grounds that it would probably be detrimental to the specific antigen-antibody interaction.

vii) The use of $(FAB)_2$ fragments of immune and non-immune IgG was considered in order to circumvent Fc binding, but the approach is technically complicated (208, 278) and would only have been attempted if the non-specific staining component could be clearly distinguished from specific staining.

2.3. Discussion.

The experiments reported on non-specificity were carried out over a period of 12 months. During this time a variety of antibodies to cyclic AMP and cyclic GMP had been produced (see immunization schedule I page 127) which specifically bound the respective nucleotide, but which showed non-specific immunofluorescence in C.N.S. tissue sections. Personal communication with Drs Floyd Bloom and Alton Steiner showed that they had not experienced problems with non-specificity in brain tissue using identical methodology, but different antibodies. This suggested that they might be employing cyclic nucleotide antibodies of higher avidity or higher titre, enabling dilution to remove any non-specific effects.

In January 1977, I was able to spend a period of three weeks at work in Dr Steiner's laboratory (University of North Carolina, U.S.A.) to evaluate different antibodies for immunofluorescent localization of cyclic nucleotides in rat cerebellum.

A number of specific rabbit cyclic AMP antibodies, raised against succinyl cyclic AMP-keyhole limpet hemocyanin, were screened, and several showed specific cyclic AMP immunofluorescence at high dilution, clearly distinct from sections treated in parallel with non-immune immunoglobulin. Since the immunoglobulin concentration of antibodies from different sources may vary, it was essential to compare each specific cyclic nucleotide antibody with non-immune immunoglobulin at the same protein concentration. This is an important point, since not only may pre-immune sera have lower concentrations of immunoglobulin than immune sera (279), but pre-immune sera may not always be collected and therefore available for comparison.

I was also able to examine a variety of specific rabbit cyclic GMP antibodies raised against succcinyl cyclic GMP-KLH immunogen, produced by Dr Steiner's group. Out of the antibodies tested, three showed positive staining distributed along fibres, whilst one antibody also showed staining of capillary walls; with all these antibodies, staining was observed at high dilution and could be clearly distinguished from staining with non-immune immunoglobulin. The cyclic nucleotide antibodies selected were among those employed by Steiner's group for the localization of cyclic AMP and cyclic GMP in tissues such as liver, intestine and testis (305).

My visit to Dr Steiner's laboratory demonstrated that not only cyclic AMP, but also cyclic GMP could be localized in cerebellum with contrasting distribution. Being able to select positive staining antibodies which could be employed at high dilution, eliminated problems of non-specific immunofluorescence. Experiments carried out with these cyclic nucleotide antibodies are considered in greater detail in subsequent chapters.

Returning to Edinburgh, positive staining was confirmed in the laboratory with samples of Dr Steiner's antibodies which had been selected, and an immunization programme was initiated in ten rabbits with succinyl cyclic GMP-protein immunogen , in the hope of producing successful antibodies for immunofluorescence. It was decided to concentrate on producing cyclic GMP antibodies, and monitor the bleeds from each rabbit during immunization with radioimmunological techniques, to determine which of the characteristics (titre, avidity or cross-reactivity), were required for successful localization of cyclic GMP in C.N.S. tissue sections.

CHAPTER III

PRODUCTION OF ANTIBODIES TO CYCLIC NUCLEOTIDES AND SELECTION FOR USE IN IMMUNOHISTOCHEMICAL STUDIES APPLIED TO THE C.N.S.

CHAPTER III

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1. FACTORS AFFECTING ANTIBODY PRODUCTION

1.1. Species

Rabbits and guinea-pigs are generally used for production of antibodies, although many other species have been used including horse, goat, sheep, chicken and mouse (280). Certain strains of animals may sometimes be superior to others e.g. New Zealand white rabbits are recommended (212), and inbred strains with a restricted gene pool may be particularly successful (281). In general, a large number of animals are immunized in the hope of obtaining one or two antisera with high titre, avidity and specificity. In this respect small animals are preferable in that a large number may be used in one experiment, although large animals naturally produce greater volumes of antisera.

1.2. Adjuvant

Not all macromolecules are equally effective in eliciting a good immune response. Various local irritants and other 'adjuvants' have been introduced which overcome this difficulty to some extent (282). The adjuvants probably act by boosting the immune system and inducing local inflammation with proliferation of macrophages, which brings them into contact with antigen. Adjuvants also cause a local granulomatous lesion, which may act as a focus for antibody production (282). The adjuvant enables the antigen to be present in long-lasting form at the site of injection, helping to prevent rapid degradation and any direct physiological effect of the immunogen itself, on the animal. Adjuvants are generally made from mineral oils and mycobacteria which are then emulsified with antigen, although antigen adsorbed onto alum has been used (283). The most common adjuvant is Freund's mineral oil either 'complete' (containing killed, dried mycobacterium

tuberculosis) or 'incomplete' (without mycobacteria), both forms being commercially available and employed as water-in-oil emulsions (284).

The physical form of antigens which do not require adjuvants may also be important for eliciting a good immune response e.g. bovine γ globulin is a good immunogen in mice in the aggregated form, but when ultra-centrifuged to be freed from aggregates, it is ineffective (193).

1.3. Route of immunization

It is arguable which route is best for successful immunization, although it is generally agreed that the intra-venous route is not effective (212). Commonly, oily adjuvant-immunogen emulsions are injected intra-dermally, sub-cutaneously or intra-muscularly. The first two procedures have the advantage that whilst small abscesses usually form and ulcerate at the site of injection (usually the upper region of the back), the animals do not seem unduly troubled by this. Foot-pad injection, despite its reported efficacy, should be avoided since animals are obviously in considerable pain with swollen, tender feet (285).

The multiple intra-dermal injection procedure of Vaitukaitis et al (286) is a simple technique reported to be successful in producing near maximal response after a single injection at approximately 40 sites on the animals back. Whilst animals develop extensive skin ulceration, they are reported to remain comfortable and in good condition with this procedure.

1.4. Dosing regimen

After the first injection the antiserum titre rises slowly and forms a plateau after about 4 - 6 weeks, from which time a variable slow decline occurs (212). The secondary immune response produced by a series of booster injections may, or may not, increase the titre. The avidity of antiserum generally increases with time, although it may fall off or increase suddenly, during a series of boosters. There is evidence that high avidity is favoured by spacing boosters widely, and good avidity has been shown to return when an animal has been rested for periods of several months, after a series of shorter-spaced injections (212).

Small doses of immunogen, rather than large doses, are used for injection. Large doses have the capacity to induce tolerance in cells which contain, and potentially secrete, high avidity antibodies, which could prevent the cells from further proliferation (287).

The primary antibody response produces antibody of the IgM type, whilst long term immunization with booster injections gives antibody almost entirely of the IgG type (282).

Peak IgG antibody response occurs 10 - 14 days after booster injections of oily emulsions, and animal bleeds are therefore harvested at this time (285).

2. PRODUCTION OF ANTIBODIES TO HAPTENS

Whilst molecules of low molecular weight are generally not immunogenic, the classical immunochemical experiments of Landsteiner (288) showed that specific antibodies could be raised to well-defined small molecules when coupled to proteins, and used as antigens. He defined these small molecules as haptens, 'specific protein-free substances

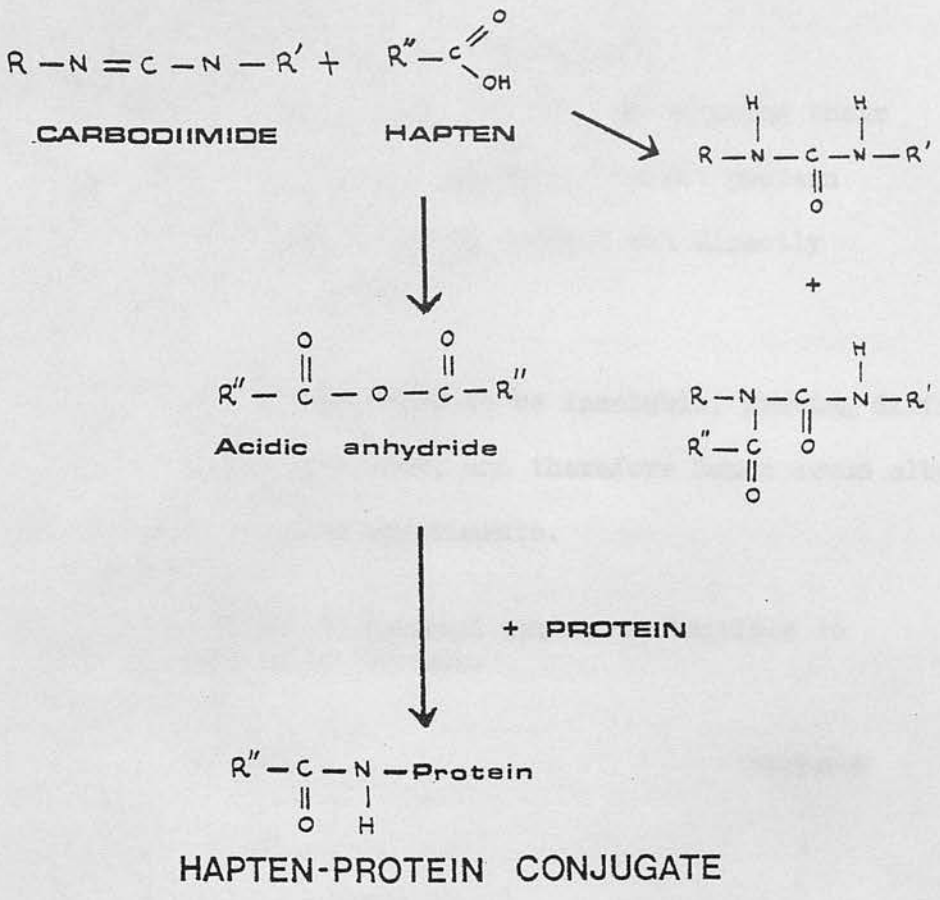


Figure 15 Mechanism for coupling hapten and protein using carbodiimide

which, although reactive'in vitro', induced none or only slight, antibody response.'

Haptens are covalently coupled to high molecular weight 'carriers' e.g. serum albumin (molecular weight~69,000), keyhole limpet hemocyanin (molecular weight ~ 2.7×10^6), for immunization. Common methods of coupling form peptide or C=N bonds.

Carbodiimide coupling is shown in figure 15, and is a widely used condensation reaction generating a peptide linkage between protein and carboxyl groups of the hapten (289).

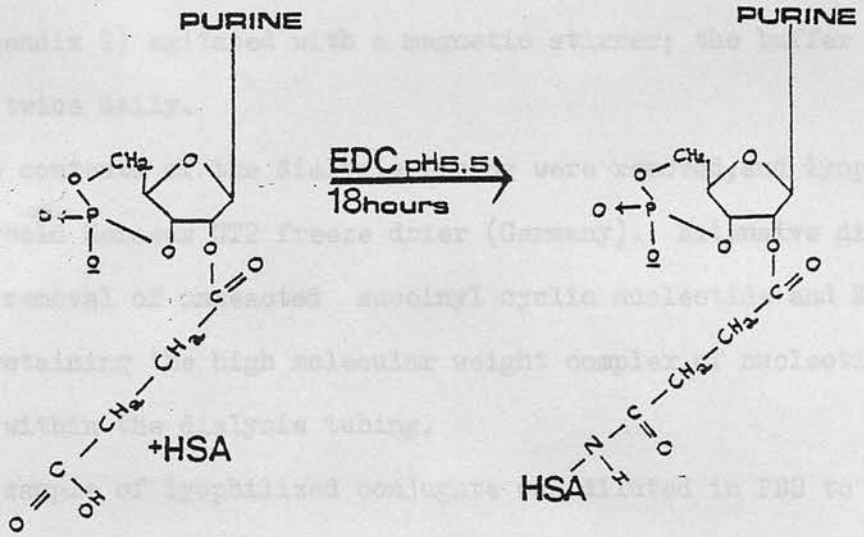
3. PRODUCTION OF SPECIFIC ANTIBODIES TO CYCLIC AMP AND CYCLIC GMP - METHODOLOGY

3.1. Preparation of hapten-protein conjugates.

Cyclic nucleotides were rendered immunogenic by coupling their succinylated derivatives to a high molecular weight protein carrier, the coupling procedure being carried out directly according to Steiner et al (230).

Keyhole limpet hemocyanin was found to be insoluble, proving difficult to work with as a dark-grey powder, and therefore human serum albumin was employed for all coupling experiments.

Figure 16 Coupling of succinyl cyclic nucleotides to human serum albumin.



Coupling method:

The reaction was carried out in glass vials, as used for scintillation counting.

i) 30mg of human serum albumin (crystallized form, Sigma Chemical Co.) were dissolved in 3ml of distilled water.

ii) 15mg of succinyl cyclic AMP or succinyl cyclic GMP (supplied as the free acids from Boehringer Mannheim, Sussex) were added, the pH then being adjusted to 5.5.

iii) 15mg of 1-ethyl-3 (3,dimethyl-aminopropyl)-carbodiimide hydrochloride, EDC (Sigma Chemical Co.), were dissolved in the solution and the pH was rapidly re-adjusted to 5.5.

iv) The vial was stoppered, wrapped in aluminium foil to prevent access of light, and incubated for approximately 18 hours at room temperature.

v) The solution was transferred to Visking 10mm width dialysis tubing (Gallenkamp, Glasgow) which had been previously boiled in distilled water to remove traces of glycerin. Dialysis was carried out for 4 days at 4°C, against 5 litres of phosphate-buffered saline (see appendix 2) agitated with a magnetic stirrer; the buffer was changed twice daily.

vi) The contents of the dialysis tubing were removed, and lyophilized in a Leybold Heraeus GT2 freeze drier (Germany). Extensive dialysis ensured removal of unreacted succinyl cyclic nucleotide and EDC, whilst retaining the high molecular weight complex of nucleotide and protein within the dialysis tubing.

vii) A sample of lyophilized conjugate was diluted in PBS to a concentration of 0.5mg/ml, and examined in an ultra-violet spectrophotometer (Pye Unicam, SP500), scanning from 190-350nm against PBS, and against HSA at the same concentration, to confirm formation of the conjugate (see figure 17). Succinyl cyclic GMP-HSA conjugate exhibited maximum absorbance at 255nm whilst succinyl cyclic AMP-HSA conjugate showed a maximum at 257nm. Unconjugated HSA however, showed maximum absorbance at 280nm, whilst EDC showed no absorption maxima in these regions. Steiner et al (230) have calculated that 5-6 succinyl cyclic nucleotide molecules couple to each albumin molecule, with this procedure.

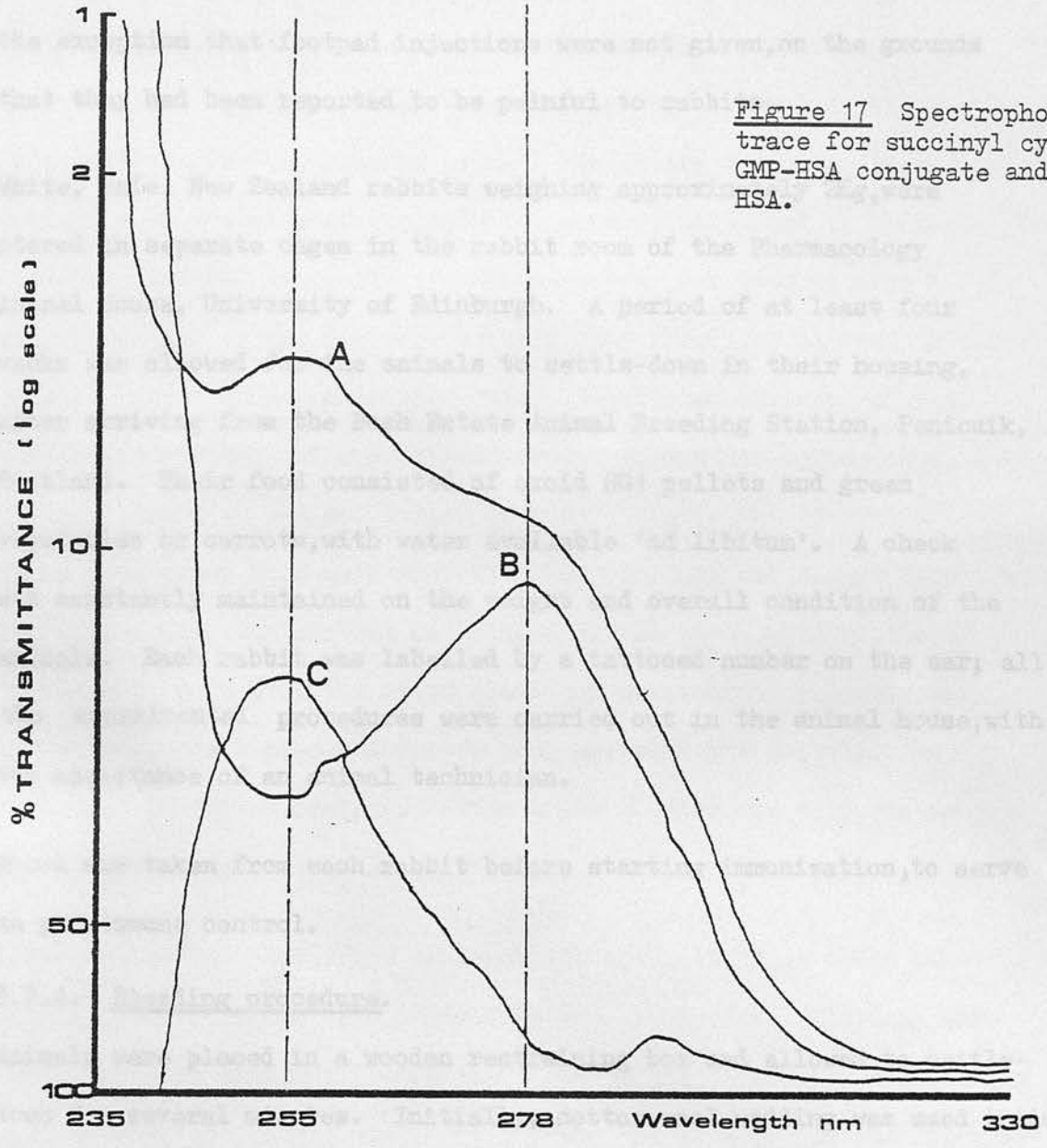


Figure 17 Spectrophotometer trace for succinyl cyclic GMP-HSA conjugate and for HSA.

A - 0.5mg/ml succinyl cyclic GMP-HSA conjugate vs. PBS

B - 0.5mg/ml HSA vs. PBS

C - 0.5mg/ml succinyl cyclic GMP-HSA conjugate vs. 0.5mg/ml HSA

3.2. Immunization of rabbits.

Immunization was carried out according to Steiner et al (230), with the exception that footpad injections were not given, on the grounds that they had been reported to be painful to rabbits.

White, male, New Zealand rabbits weighing approximately 2Kg, were stored in separate cages in the rabbit room of the Pharmacology Animal House, University of Edinburgh. A period of at least four weeks was allowed for the animals to settle-down in their housing, after arriving from the Bush Estate Animal Breeding Station, Penicuik, Scotland. Their food consisted of oxoid SG1 pellets and green vegetables or carrots, with water available 'ad libitum'. A check was constantly maintained on the weight and overall condition of the animals. Each rabbit was labelled by a tattooed number on the ear; all the experimental procedures were carried out in the animal house, with the assistance of an animal technician.

Blood was taken from each rabbit before starting immunization, to serve as pre-immune control.

3.2.A. Bleeding procedure.

Animals were placed in a wooden restraining box and allowed to settle-down for several minutes. Initially, cotton wool wadding was used inside the box to prevent the rabbits moving, but this resulted in the death of two rabbits, due to rapid twisting of the head which caused breakage of the neck. A plastic restraining box with adjustable sides (Forth Tech, Scotland) was subsequently employed, which ensured that the rabbits were unable to move whilst being bled. Fur was plucked from the upper side of one of the rabbits ears, and the marginal ear vein was located. Petroleum jelly (Chesebrough-Ponds Ltd, London)

was smeared onto the edge of the ear to prevent clotting of drawn blood on the fur. The remainder of the ear was dabbed with cotton wool soaked in xylene, to cause local irritation and dilatation of the vein. Care was taken to ensure that the xylene did not come into contact with the area to be cut. An artery clip was placed posterior to the site of incision to cause a 'build-up' of blood. A sterile scalpel blade (No. 11 Swann-Morton) was used to make a small incision in the exposed ear vein, and blood was allowed to trickle into a glass collection tube. Accidental incision of the underlying artery, which sometimes occurred, was not serious and helped to quicken the collection of blood; approximately 30ml of blood were drawn from each rabbit. When bleeding was almost complete the artery clip was removed and placed on the other side of the incision to help stop the blood flow. On completion, the clip was placed on a small piece of cotton wool covering the incision, to assist clotting and healing. After a few minutes the clip was removed and the rabbit was returned to its cage.

After collecting blood from each rabbit, the collection tubes were sealed with 'parafilm' and removed to the laboratory. Clotting was facilitated by either allowing the blood to stand for one hour at room temperature, or leaving undisturbed overnight at 4°C. It was important not to exceed these clotting times, to prevent haemolysis. Fibrous clots were removed or loosened from the sides of the collection tubes. Serum was separated from corpuscles by centrifugation at 2,500 r.p.m. for 30 minutes at 4°C (MSE Mistral 2L centrifuge). A Pasteur pipette was used to remove serum from each tube, which was then transferred to clean, labelled tubes. Approximately 15ml of serum were obtained from 30ml of blood. 5ml of serum were stored frozen in the deep-freeze

at -25°C, whilst the remaining 10ml were used directly for preparation of the immunoglobulin fraction (see page 129)

3.2.B. Injection procedure.

The immunogen, lyophilized succinyl cyclic nucleotide-human serum albumin conjugate, was dissolved in PBS and added to Freund's adjuvant, in a small glass beaker. The mixture was vigorously mixed for 4 - 5 minutes using a Polytron mixer (Northern Medical Supply Ltd, Great Britain) until a thick white emulsion formed. The beaker was kept constantly cool with a damp cloth during this procedure. It was essential to add the immunogen to the oily adjuvant, rather than the other way around, in order to form a stable emulsion (212). The stability was checked by ensuring that the emulsion remained in a single phase when stored overnight at 4°C. An immunogen-adjuvant ratio of 1:1 was initially used, but produced a very thick emulsion which proved difficult to pack into the syringe for injection; ratios of 1:2 and 1:3 were subsequently found to be more satisfactory (285). Approximately double the quantity of emulsion required was made, since regardless of the immunogen-adjuvant ratio, it proved impossible to transfer all the emulsion from beaker to syringe.

Rabbits were firmly held on a flat table, ensuring that they were not allowed to move during injection. For the primary injection, immunogen was injected in Freund's 'complete' adjuvant (Miles Laboratories, Slough, Buckinghamshire). The emulsion was injected intra-dermally into approximately ten sites in the upper shaved region of the animals back. A glass 1ml syringe was found preferable to the plastic disposable type for injection. Approximately 0.3ml of emulsion were injected at each site, a total of 3ml being injected per rabbit. Disposable 21 guage needles were used for injection.

Sub-cutaneous booster injections in Freund's 'incomplete' adjuvant were given at two or three sites in the shaved regions of the animals back, using a glass 1ml syringe with disposable 25 guage needles. Approximately 0.5ml of emulsion were given at each site, forming small blisters under the skin. Animals were returned to their cages after injection.

Tables 1 and 2 summarize the immunization schedules used for production of antibodies to cyclic nucleotides. Individual bleeds are labelled, since they are referred to individually later in the text.

In schedule I (Table 1), rabbits 4 and 7 died, due to their necks breaking during bleeding, whilst in schedule II (Table 2), rabbits 3 and 4 developed ear infections, probably as a result of the routine syringing procedure, and were destroyed on humane grounds. All the other rabbits remained in good health during the course of immunization.

In schedule I (Table 1), the first injection employed an immunogen-adjuvant ratio of 1:1 whilst booster injections used a ratio of 1:3. In schedule II (Table 2), the first injection used a ratio of 1:2 whilst for booster injections the ratio was 1:1.

In schedule I (Table 1) rabbits were rested for four months between the fourth and fifth bleeds (table indication ***).

Table 1 Initial experiment for production of antisera to cyclic AMP and cyclic GMP (Immunization schedule I, 1976)

	Day number	SUCCINYL cAMP-HSA IMMUNOGEN			SUCCINYL cGMP-HSA IMMUNOGEN		
		Rabbit 3	Rabbit 4	Rabbit 5	Rabbit 6	Rabbit 7	Rabbit 8
PRE-IMMUNIZATION BLEED	0		DIED				
FIRST INJECTION 1mg	1						
BLEED	36	3B1		5B1	6B1	DIED	8B1
BOOSTER 250μg	52						
BLEED	65	3B2		5B2	6B2		8B2
BOOSTER 250μg	94						
BLEED	104	3B3		5B3	6B3		8B3
BOOSTER 250μg	129						
BLEED	140	3B4		5B4	6B4		8B4
*** BOOSTER 250μg	255						
BLEED	294	3B5		5B5	6B5		8B5

Table 2 Production of antisera against succinyl cyclic GMP-HSA immunogen (Immunization schedule II, 1977)

	Day number	RABBIT NUMBER									
		A1	A2	A3	A4	A5	A6	A7	A8	A9	A10
PRE-IMMUNIZATION BLEED	0										
FIRST INJECTION 1mg	1										
BOOSTER 200μg	28										
BLEED	38	A1B1	A2B1	DIED	A4B1	A5B1	A6B1	A7B1	A8B1	A9B1	A10B1
BOOSTER 200μg	63										
BLEED	73	A1B2	A2B2		A4B2	A5B2	A6B2	A7B2	A8B2	A9B2	A10B2
BOOSTER 200μg	98										
BLEED	108	A1B3	A2B3		A4B3	A5B3	A6B3	A7B3	A8B3	A9B3	A10B3
BOOSTER 200μg	126				DIED						
BLEED	136	A1B4	A2B4			A5B4	A6B4	A7B4	A8B4	A9B4	A10B4
BOOSTER 200μg	161										
BLEED	171							A7B5			A10B5

3.3. Preparation of immunoglobulin fraction from serum

Immunoglobulin fractions were prepared from sera not only to concentrate the antibody-containing fraction, but also to remove cyclic nucleotide phosphodiesterase activity present in serum, which might destroy the cyclic nucleotides in tissue sections, or during the radioimmunological techniques for estimation of antibodies. Free circulating cyclic nucleotides would also be removed from serum by this fractionation procedure (see page 169).

Ammonium sulphate fractionation of serum at 40% concentration is a simple and effective means of preparing a crude immunoglobulin fraction from pre-immune and immune sera, and is often employed prior to chromatographic preparation of highly purified IgG from serum (265).

Method: 400g of ammonium sulphate (especially low in heavy metals) were dissolved in 500ml of distilled water with constant stirring at 70-80°C. The solution was stirred for about 20 minutes, covered, and allowed to cool to room temperature, whilst needle-like crystals formed at the bottom of the beaker. After cooling, the saturated supernatant was decanted into another beaker, and the pH adjusted to 7.4 with ammonium hydroxide (28%) and sulphuric acid (50%). The solution was stored at 4°C, and filtered prior to use.

The 'salting-out' procedure of immunoglobulin from serum (carried out according to Nairn, reference 194) was performed 'on ice' in the laboratory, after pre-cooling all the reagents.

Saturated ammonium sulphate solution equal to $\frac{2}{3}$ of the serum volume, was added at the rate of approximately one drop per second, in a glass test tube. The mixture was stirred gently with a glass rod, as a thick

white precipitate formed. Stirring was continued for a short time before allowing the mixture to stand 'on ice' for 30 minutes to obtain maximum precipitation. The mixture was centrifuged at 2,500r.p.m. for 30 minutes at 4°C (MSE Mistral 2L centrifuge). The resulting supernatant was discarded and the precipitate dissolved in PBS to give a total volume equal to half the original serum volume.

The immunoglobulin fraction, dissolved in PBS, was dialyzed against this buffer for three days at 4°C with two changes of buffer per day (see page 121). On completion of dialysis, one drop of 1M barium chloride was added to one drop of the immunoglobulin fraction to test for SO_4^{2-} ions. A white turbid solution, clearing on acidification with hydrochloric acid, would have indicated the presence of sulphate ions, and therefore incomplete dialysis. Complete removal of ammonium sulphate was always found with this dialysis technique however.

The immunoglobulin fraction was clarified by centrifugation at 13,000g for three minutes (Eppendorf centrifuge), divided into aliquots and stored frozen at -25°C. Aliquoting prevented constant freeze-thawing which can result in aggregate formation, a possible cause of non-specificity in immunofluorescence (235).

To confirm that the fractionation technique resulted in an enrichment of IgG in the crude immunoglobulin fraction, the percentage of protein representing IgG in the immunoglobulin fraction was calculated. IgG values were obtained using a Miles radial immunodiffusion kit for rabbit IgG (Miles Laboratories, Slough, Buckinghamshire), and protein values were determined by the method of Lowry et al (see appendix 5). Pre-immunization bleeds and antiserum A7B2 were found to contain approximately 10% of total protein as IgG, whereas crude immunoglobulin fractions of these and selected other bleeds (also from Dr Steiner's laboratory), contained approximately 50% of protein as IgG.

4. DETERMINATION OF THE CHARACTERISTICS OF CYCLIC NUCLEOTIDE ANTIBODIES USING RADIOIMMUNOLOGICAL TECHNIQUES.

4.1. Introduction

As already noted in chapter II, only certain cyclic nucleotide antibodies appear to be suitable for use in the immunfluorescent localization of cyclic AMP and cyclic GMP in C.N.S. tissue sections. A number of characteristics of these antibodies may contribute to their usefulness. High titre antibodies might be of importance, so that a high concentration of antibody would be available for binding on a tissue section. Alternatively, high avidity antibodies, by binding 'tightly' to cyclic nucleotides in the tissue, could be essential since they would not be easily removed during the buffer washing procedures. Finally, the specificity of cyclic nucleotide antibodies might be a critical factor, a positive staining antibody possibly being highly selective for one nucleotide, and showing minimal cross-reactivity to closely related molecules in the tissue.

In order to study these characteristics, with a view to selection of antibodies as immunohistochemical reagents, high sensitivity radioimmunlogical techniques were employed to study the binding of antibody to nucleotide in solution. Prior to starting my work in Edinburgh, cyclic nucleotide antibodies had been produced in the laboratory by Mr T Attree and screened for titre in binding assays employing $[^{3}H]$ cyclic nucleotide tracers. Whilst Steiner et al (230, 231) had employed $[^{125}I]$ tyrosine methyl esters of succinyl cyclic nucleotides as high specific activity tracers, cyclic nucleotides with tritium incorporated directly into the molecule have recently become commercially available, at high specific activity. Whilst these tracers

still have lower specific activity than [^{125}I] tracers, direct labelling on the nucleotide rather than iodination of a derivative produces a molecule with higher affinity for antibody (see page 140). Tritiated tracers also offer several practical advantages over iodinated tracers(290); they are more stable over long periods of time, and therefore do not require periodic synthesis as do iodinated compounds. Being 'soft' β emittors, tritiated tracers are also safer to handle than iodinated compounds which emit γ radiation.

The existing binding assay was initially employed to measure the titre of cyclic AMP and cyclic GMP antibodies prepared according to immunization schedule I (1976). The assay technique and results, employing 'second antibody' precipitation to separate antibody-bound and free cyclic nucleotide, are discussed, followed by modification for measurement of not only titre, but also avidity and cross-reactivity of cyclic GMP antibodies prepared by immunization schedule II (1977).

4.2. Binding of [^{3}H] cyclic AMP and cyclic GMP by dilutions of cyclic nucleotide antibodies prepared according to immunization schedule I - 'second antibody' technique.

All operations in the laboratory were carried out 'on ice' in 1.3ml plastic 'Eppendorf' tubes. Automatic 'Eppendorf' pipettes were used to dispense reagents.

i) 0.05M sodium acetate, pH 6.2, was employed as the assay buffer. The buffer was stored at 4°C for not more than a few days, since longer storage resulted in cloudiness due to bacterial formation.

ii) Rabbit immunoglobulin fractions of cyclic nucleotide antisera were diluted with buffer in dilutions from 1:10 to 1:100, and centrifuged at 13,000g for 3 minutes (Eppendorf centrifuge) to remove any aggregates. 200μl of centrifuged supernatant were placed in

Eppendorf tubes. Duplicates were made for each dilution of antibody.

iii) 1μl of [^{3}H] cyclic AMP or [^{3}H] cyclic GMP was diluted with 2.5ml of buffer. Labelled cyclic nucleotides were obtained from the Radiochemical Centre (RCC), Amersham, Buckinghamshire ; TRK 304 [8-^{3}H] adenosine 3',5'-cyclic phosphate, ammonium salt, specific activity of free acid-27.5Ci/m mole, concentration 1mCi/ml. TRK 392 [8-^{3}H] guanosine 3',5'-cyclic phosphate, ammonium salt, specific activity of free acid-21.0 Ci/m mole, concentration 1mCi/ml. Labelled cyclic nucleotides were received from RCC in 50% ethanol, and stored at -25^{o}C as recommended, being diluted with buffer prior to use.

50μl of either diluted [^{3}H] cyclic nucleotide were added to the antibody in the Eppendorf tubes, briefly vortex mixed , and incubated for four hours at 4^{o}C.

iv) An appropriate dilution of high titre donkey anti-rabbit serum (code no. RD.17, Lot no. K1953B, Wellcome Laboratories, Beckenham, Kent) was added in a volume of 50μl. The reaction tubes were briefly vortex mixed, and incubated at 4^{o}C for a further 18-20 hours in order to precipitate rabbit antibody.

v) The reaction tubes were centrifuged in batches of twelve at 13,000g,for three minutes.

vi) The supernatants were carefully removed and transferred to glass scintillation vials in preparation for radioactivity 'counting' .

vii) 200μl of buffer were added to the tubes followed by vortex mixing to wash the precipitates. Centrifugation was then repeated for a further six minutes.

viii) The resulting supernatants were transferred to scintillation

vials in preparation for radioactivity counting.

ix) The remaining precipitates were dissolved by adding 100μl of 98% formic acid, vortex mixing, and allowing to stand at room temperature for 30 minutes. 100μl of distilled water were then added to dilute the acid. After mixing, the solubilized precipitates were transferred to scintillation vials, in preparation for radioactivity counting.

x) 10ml of NE 260 micellar liquid scintillation cocktail (Nuclear Enterprises, Edinburgh) were added to each of the vials, which were then stoppered and thoroughly mixed. A period of one hour was allowed to reduce any chemiluminescence, prior to counting of radioactivity (291). Samples were counted for four minutes each using an automatic Nuclear Chicago scintillation counter. Machine 'background' was calculated by counting a 200μl buffer sample with scintillant; this value was substracted from each reading. Counting efficiency for tritium was calculated at 40%.

The percentage of total radioactivity bound by each dilution of antibody was calculated according to the formula:

$$\% \text{ radioactivity bound} = \frac{C}{A+B+C}$$

where:

A = number of counts from supernatants fraction
B = number of counts from 'wash' fraction
C = number of counts from precipitate

Non-specific binding was calculated as the percentage radioactivity bound from the immunoglobulin fraction taken from unimmunized rabbits.

4.2.A. Notes on assay

The labelled cyclic nucleotides chosen, had the highest specific activity commercially available, when these experiments were carried out. 0.73 p moles of [^{3}H] cyclic AMP and 0.95 p moles of [^{3}H] cyclic GMP were added per assay tube (approximately 17,000 and 13,000 counts/minute respectively). Samples were counted for four minutes each to reduce statistical counting errors and were discarded if duplicate variation exceeded 5%.

Experiments were carried out to determine the quantity of 'second antibody' required to precipitate the maximum amount of radioactivity, for different dilutions of cyclic nucleotide antibodies (see figure 18). At low dilutions of cyclic nucleotide antibody, increasing amounts of 'second antibody' were required for maximal precipitation. It was important to adjust 'second antibody' concentrations for each dilution, since decreased binding would result using levels above or below the optimum.

4.2.B. Results

Tables 3 and 4 show the effect of dilution of immunoglobulin fractions, on percentage radioactivity bound, by bleeds taken from rabbits 3 and 5 (immunized with succinyl cyclic AMP-HSA), and rabbits 6 and 8 (immunized with succinyl cyclic GMP-HSA). Non-specific binding of [^{3}H] cyclic nucleotides by non-immune immunoglobulins at a dilution of 1:50, was found to be approximately 1.0%.

Antibodies with similar titre were found in four rabbits injected with succinyl cyclic AMP-HSA and succinyl cyclic AMP-KLH, and three rabbits injected with succinyl cyclic GMP-HSA conjugate, previously produced in the laboratory by Mr T Attree.

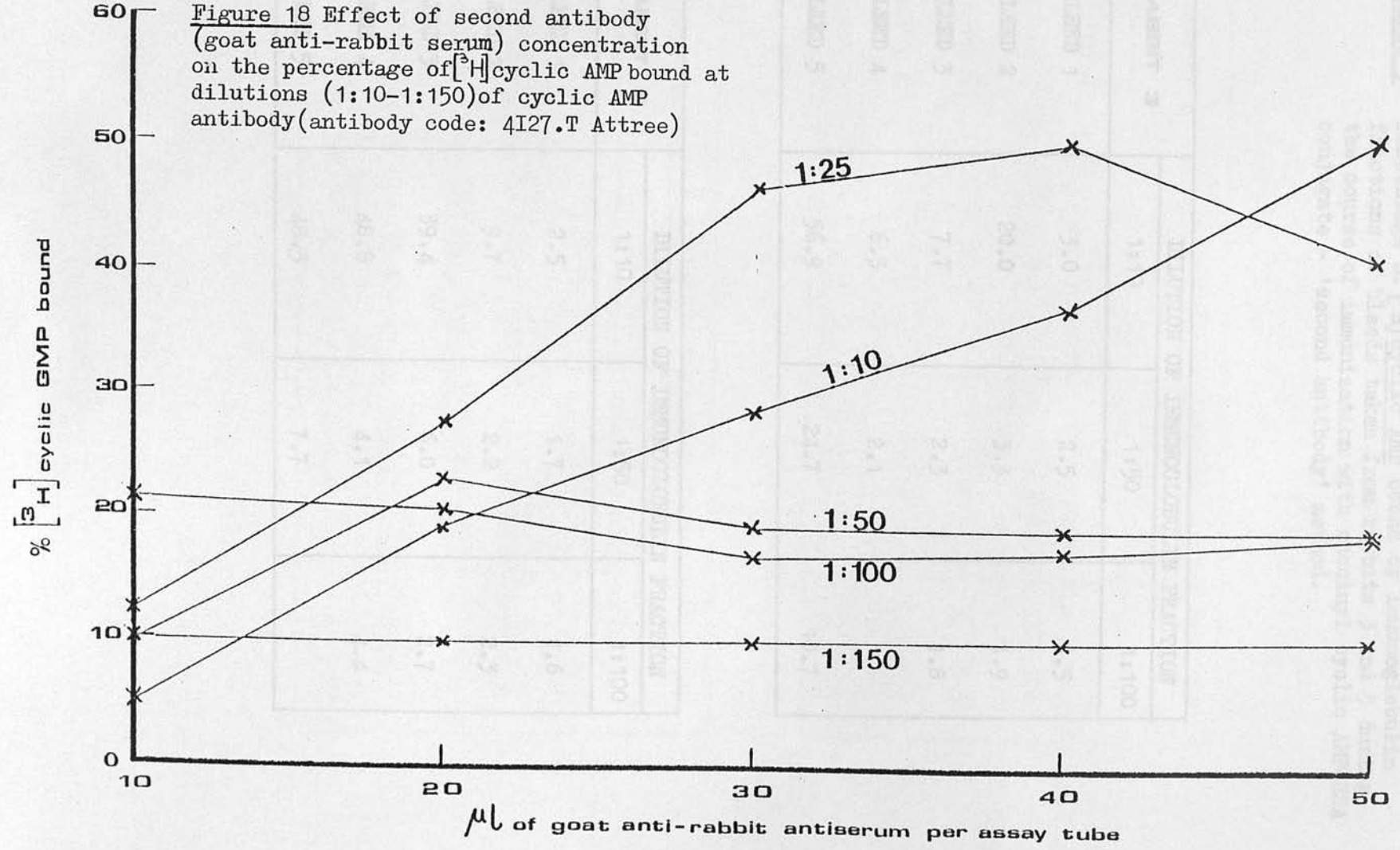


Figure 18 Effect of second antibody (goat anti-rabbit serum) concentration on the percentage of [^{3}H]cyclic AMP bound at dilutions (1:10-1:150) of cyclic AMP antibody (antibody code: 4I27.T Attree)

Table 3 Percentage of [^{3}H] cyclic AMP bound by immunoglobulin fractions of bleeds taken from rabbits 3 and 5 during the course of immunization with succinyl cyclic AMP-HSA conjugate - 'second antibody' method.

RABBIT 3	DILUTION OF IMMUNOGLOBULIN FRACTION		
	1:10	1:50	1:100
BLEED 1	3.0	2.5	1.5
BLEED 2	20.0	3.3	1.9
BLEED 3	7.7	2.3	1.8
BLEED 4	6.3	2.1	
BLEED 5	56.9	24.7	10.7

RABBIT 5	DILUTION OF IMMUNOGLOBULIN FRACTION		
	1:10	1:50	1:100
BLEED 1	2.5	1.7	1.6
BLEED 2	9.7	2.2	2.3
BLEED 3	39.4	6.0	2.7
BLEED 4	48.8	4.1	4.4
BLEED 5	48.3	7.7	

Table 4 Percentage of [^{3}H] cyclic GMP bound by immunoglobulin fractions of bleeds taken from rabbits 6 and 8 during the course of immunization with succinyl cyclic GMP-HSA conjugate - 'second antibody' method

RABBIT 6	DILUTION OF IMMUNOGLOBULIN FRACTION		
	1:10	1:50	1:100
BLEED 1	14.5	3.7	2.2
BLEED 2	2.9	1.9	1.9
BLEED 3	44.3	12.6	5.5
BLEED 4	57.6	35.4	16.3
BLEED 5	61.8	41.9	21.1

RABBIT 8	DILUTION OF IMMUNOGLOBULIN FRACTION		
	1:10	1:50	1:100
BLEED 1	30.4	5.8	2.6
BLEED 2	30.9	6.2	3.3
BLEED 3	29.9	5.2	2.9
BLEED 4	65.8	29.0	14.0
BLEED 5	56.9	26.3	12.9

This pilot study demonstrated that antibodies to cyclic AMP and cyclic GMP could be produced in rabbits with variations in titre not only between different animals, but also between different bleeds from the same animal, with a general increase on repeated boosting.

In an attempt to produce antibodies of higher titre, and to carry out a study of variations in titre, avidity and specificity during immunization, a complete study was initiated using ten rabbits immunized with succinyl cyclic GMP-HSA conjugate.

4.2.C Discussion

A number of factors suggested that the binding assay should be modified before the more extensive studies were performed on the antibodies from the ten rabbits, injected according to immunization schedule II. The requirement for 200μl of cyclic nucleotide antibody consumed large amounts of antibody in the binding studies. Secondly, 50μl of undiluted 'second antibody' were required per assay tube for maximal precipitation of radioactivity with low titre antibody, which proved expensive for large numbers of tubes. Finally, 0.95 p moles of [^{3}H] cyclic GMP were used per assay tube, which would require the assay to be significantly scaled-down for development of a radioimmunoassay to approach the sensitivity to that reported by Steiner et al i.e. $\sim$ 0.01 p moles (231).

The announcement of a specific and sensitive radioimmunoassay kit for cyclic GMP by the Radiochemical Centre, Amersham, Buckinghamshire (333), formed the basis for a modified binding assay to measure the titre of cyclic GMP antibodies produced in the laboratory. The kit employed 50μl of diluted specific cyclic GMP antiserum in a total incubation volume of 200μl. The most important development was the

use of an ammonium sulphate step for precipitation of antibody-bound antigen, rather than 'second antibody' precipitation. The salt precipitation technique is cheap and rapid, preventing the lengthy 18-20 hour second incubation step. Cyclic nucleotides and other small molecules e.g. peptides, lend themselves particularly well to fractional precipitation techniques (e.g. ammonium sulphate and ethanol), being soluble at concentrations of precipitant, at which immunoglobulins are completely insoluble (282). Steiner et al (231) have also adopted the ammonium sulphate technique for their iodinated radioimmunoassay, reporting similar results between this technique and the 'second antibody' method, for separating bound and free nucleotides.

The Radiochemical Centre method was adapted for use with immunoglobulin fractions of antisera, and also higher specific activity [^{3}H] cyclic GMP (31Ci/m mole) labelled on the ribose sugar. The sensitivity of the cyclic GMP kit and modified technique are close to that found with iodinated radioimmunoassay, although the specific activity of the tritiated cyclic GMP is still more than five times lower than that of the iodinated tracer. This is probably due to cyclic GMP being more effective in competing with and displacing [^{3}H] cyclic GMP from specific antibody, than in displacing an iodinated tyrosine methyl ester derivative (292).

The modified technique is described in detail for the measurement of titre, and subsequently avidity and specificity, of cyclic GMP antibodies prepared according to immunization schedule II.

4.3. Binding of [^{3}H] cyclic GMP by dilutions of antibodies prepared according to immunization schedule II - 'ammonium sulphate' technique.

Method:

All operations in the laboratory were carried out 'on ice' in 1.3ml plastic Eppendorf tubes.

i) 0.05M TRIS + 4mM EDTA, pH 7.5, was employed as the assay buffer.

ii) High specific activity radiolabelled cyclic GMP (31 Ci/m mole) was obtained from the Radiochemical Centre, Amersham, Buckinghamshire (RCC). 1µl of [ribose-5-^{3}H] cyclic GMP (TRK 499) was diluted with 8ml of assay buffer. 50µl aliquots were added to Eppendorf tubes to give a total of approximately 22,000 counts/4 minutes (approximately 0.2 p moles), per tube. [^{3}H] cyclic GMP was received from RCC in 50% ethanol and stored as recommended at -25°C, being diluted with buffer prior to use.

iii) 50µl of 'carrier' rabbit immunoglobulin (Dakopatts, Denmark; supplied by Mercia Diagnostics, Watford, England).

iv) 50µl of buffer were added to each tube.

v) 50µl of diluted antibody were added to each tube, to give a total incubation volume of 200µl. Tubes were stoppered and briefly vortex mixed. Duplicate determinations were carried out at each antibody dilution.

vi) Tubes were incubated 'on ice' for 1½ hours at 4°C.

vii) 1ml of ice-cold 60% saturated ammonium sulphate (58.5g of the salt, low in heavy metals, dissolved in 150ml of distilled water) was added to each tube; the tubes were then allowed to stand 'on ice' for five minutes.

viii) Tubes were centrifuged in batches of 12 for 3 minutes at 13,000g, using an Eppendorf centrifuge. After replacing the tubes 'on ice'

supernatants were carefully removed by suction and discarded.

ix) 1.1ml of distilled water were added to each tube, and left for at least five minutes, to dissolve the pellet.

x) Tubes were thoroughly vortex mixed. A 1ml sample was removed from each tube and transferred to glass scintillation vials, in preparation for radioactivity counting.

xi) 10ml of NE 260 micellar scintillation cocktail were added to each of the vials, which were then stoppered and thoroughly mixed. Samples were counted for four minutes each, as previously described (see page 134).

xii) Machine 'background' was calculated by counting a 1ml sample of distilled water with scintillant; this value was subtracted from each reading. The percentage of $[^3H]$cyclic GMP bound by antibody was calculated as the (number of counts bound) divided by the (total number of counts per assay tube) the product being multiplied by 100. Total counts were obtained by counting two tubes with 50µl of $[^3H]$ cyclic GMP + 1ml of distilled water + 10ml of scintillant. The mean number of total counts were divided by 1.1 to obtain counts per assay tube.

The percentage of radioactivity bound by dilutions of non-immune immunoglobulin was also calculated.

4.3.A Notes on assay

i) Separation of bound and free radioactivity.

Very low precipitation of radioactivity was found in initial experiments using $[^3H]$cyclic GMP and rabbit anti-cyclic GMP immunoglobulin. Experiments were therefore carried out with different amounts of non-immune rabbit immunoglobulin to act as

'carrier' (212), and aid precipitation of antibody-bound tracer.

The effect of carrier concentration was examined on precipitation of [^{3}H] cyclic GMP in two experiments (see figure 19). In one, a low titre antibody (8 B 5) was examined in dilutions of 1:10-1:50, whilst in the other a high titre antibody (A7 B5) was employed at 1:100-1:500. A quantity of 500μg of carrier was selected as giving maximal precipitation at all dilutions, with both types of antibody.

To ensure that the ammonium sulphate separation step was as efficient as the 'second antibody' technique previously employed, the percentage radioactivity bound with ammonium sulphate (and 500μg of carrier), was compared with that obtained using 'second antibody' at similar (<u>final</u>) dilutions to those employed in section 4.2 (see page 136). The results, shown below, demonstrate that ammonium sulphate separation is equal to, or more effective than, 'second antibody' separation, in this study:

A7 B5 (ANTIBODY DILUTION: 1:300)	% [^{3}H] cGMP bound
a) Ammonium sulphate separation (+ 500μg carrier)	36.5
b) Second antibody, final dilution 1/20	32.5
c) Second antibody, final dilution 1/10	31.3
d) Second antibody, final dilution 1/4	20.0

Note: Reaction incubated for 1½ hours at 4°C prior to addition of 'second antibody' (high titre donkey anti-rabbit serum, Wellcome Laboratories, Beckenham, Kent). Reaction time for 'second antibody' step: 18 hours, 4°C.

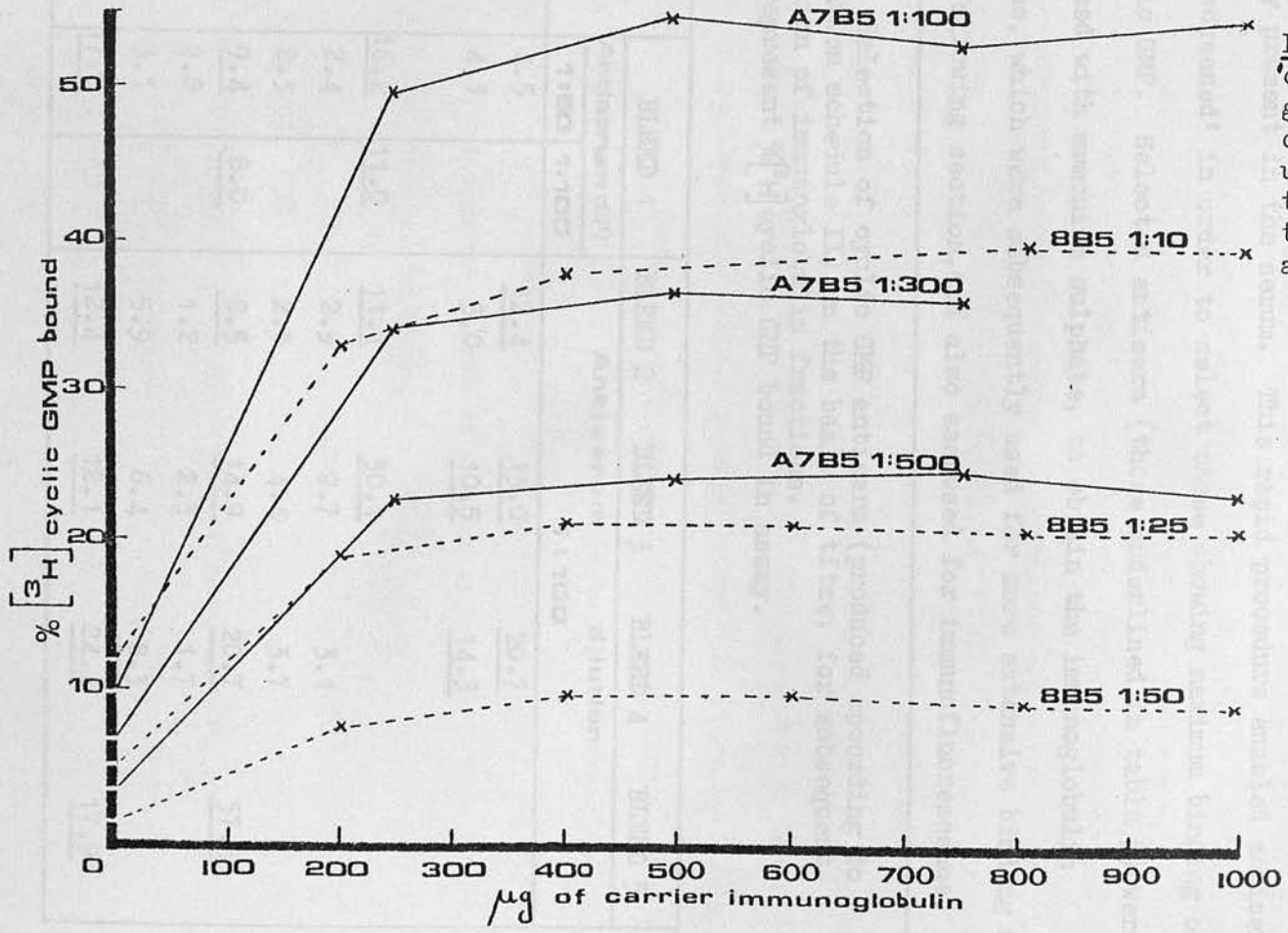


Figure 19 The effect of 'carrier' rabbit immunoglobulin concentration on bound [3H] cyclic GMP, using dilutions of high titre (A7 B5) and low titre (8 B5), cyclic GMP antibodies.

ii) Antibody selection

For selection of antibodies with highest titre produced from the ten rabbits immunized with succinyl cyclic GMP-HSA, a 1:100 dilution of unfractioned antiserum (1:50 and 1:100 dilution of bleed 1) was tested for binding, from each bleed of each rabbit. The high dilution was employed to minimize any phosphodiesterase activity present in the serum. This rapid procedure enabled antisera to be 'screened' in order to select those showing maximum binding of [^{3}H] cyclic GMP. Selected antisera (those underlined in table 5) were fractioned with ammonium sulphate, to obtain the immunoglobulin fractions, which were subsequently used for more extensive binding studies in the following section, and also assessed for immunofluorescence.

Table 5 Selection of cyclic GMP antisera (produced according to immunization schedule II) on the basis of titre, for subsequent preparation of immunoglobulin fractions.
Values represent % [^{3}H] cyclic GMP bound in assay.

RABBIT	BLEED 1		BLEED 2	BLEED 3	BLEED 4	BLEED 5
	Antiserum dilⁿ		Antiserum dilution			
	1:50	1:100	1:100			
A1	4.5		12.4	13.0	20.7	
A2	4.1		5.0	10.5	14.3	
A3						
A4	16.2	11.0	11.1	30.1		
A5	2.4		2.9	2.7	3.1	
A6	2.3		2.1	4.4	3.7	
A7	9.4	8.0	8.5	14.9	20.7	57.5
A8	2.8		1.2	2.3	1.7	
A9	3.1		5.9	6.4	8.3	
A10	17.0		12.4	12.1	24.4	17.9

4.3.B. Results

The binding of [^{3}H] cyclic GMP by 1:10 and 1:50 dilutions of immunoglobulin fractions from non-immune rabbit sera was found to be approximately 1.0%.

Selected antisera shown in table 5, were fractioned with ammonium sulphate. Graphs were constructed for each cyclic GMP antibody, of percentage radioactivity bound (ordinate) vs the dilution of immunoglobulin fraction (abscissa). Titre was selected as the dilution of antibody (undiluted protein concentration, 20mg/ml) added in 50µl, to bind 30% of added [^{3}H] cyclic GMP. In order to compare the titre of antibodies from different rabbits during the course of immunization, a graph was drawn of log inverse titre (ordinate) vs number of bleed (abscissa) for rabbits showing bleeds with titre greater than 1:15 (see figure 20).

A logarithmic rather than linear scale was used, since titre is in general distributed logarithmically (282). The graph shows the wide variation in response, but with a general trend for titre to increase during the course of immunization, but to vary in its time of onset. Rabbits A7 and A10 were given 5 booster injections in comparison to the other rabbits which received 4; it is interesting to note the different responses in titre which the last injection elicited. Rabbits A5 , A6, and A8, although boosted on four occasions, failed to produce antibody of significant titre. Steiner et al (231) have similarly reported that only 50% of rabbits showed significant binding of antiserum IgG fractions to [^{3}H] cyclic GMP, after immunization with succinyl cyclic GMP-KLH conjugate.

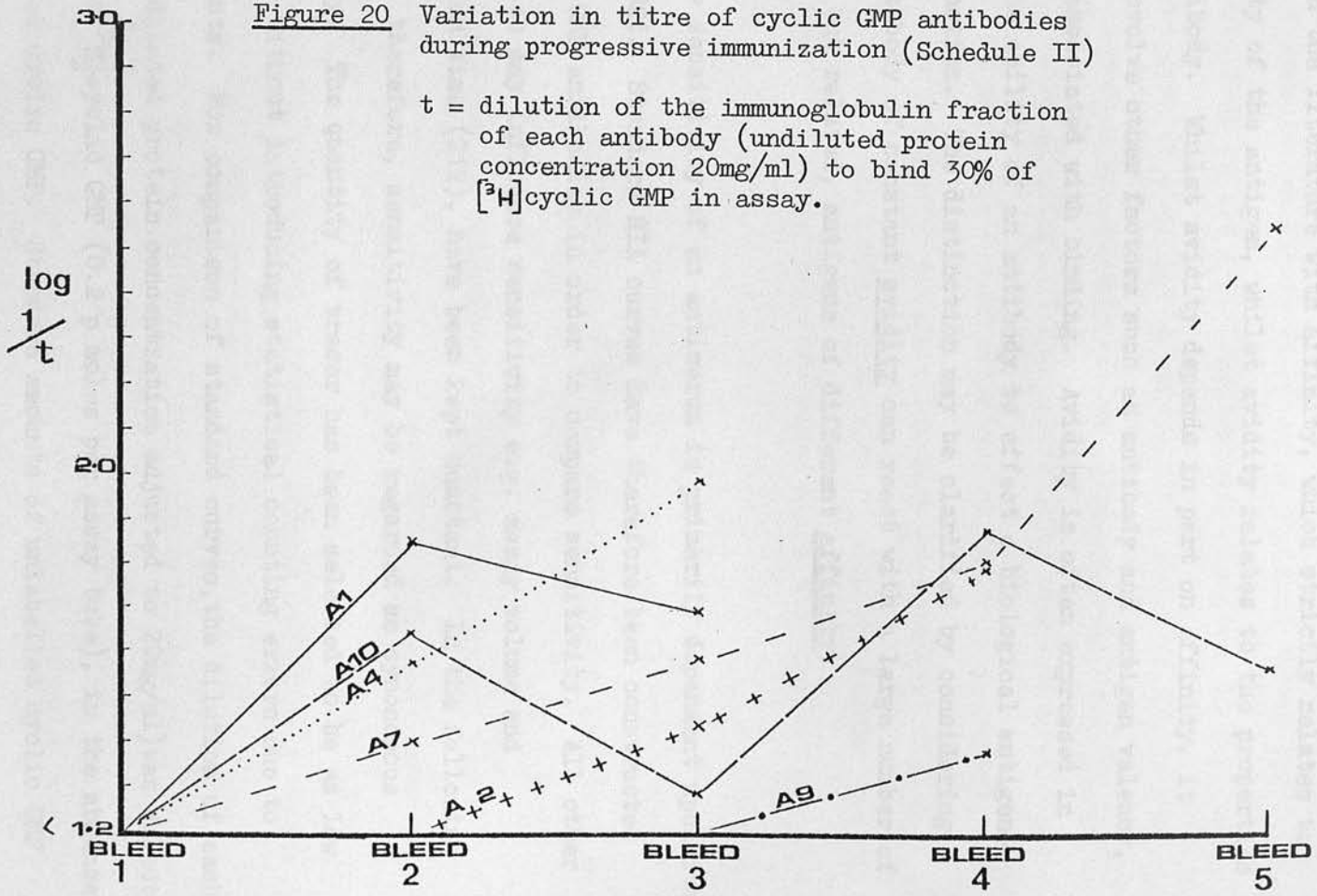


Figure 20 Variation in titre of cyclic GMP antibodies during progressive immunization (Schedule II)

t = dilution of the immunoglobulin fraction of each antibody (undiluted protein concentration 20mg/ml) to bind 30% of [^{3}H]cyclic GMP in assay.

4.4. Construction of radioimmunassay standard curves for comparison of avidity of cyclic GMP antibodies.

The titre of an antibody measures the dilution required to bind a fixed quantity of labelled antigen, and principally represents the 'amount' of specific antibody in an antiserum. Avidity is a measure of the binding energy of antibody for antigen (282); this term is often confused in the literature with affinity, which strictly relates to the property of the antigen, whilst avidity relates to the properties of the antibody. Whilst avidity depends in part on affinity, it can also involve other factors such as antibody and antigen valency, which are associated with binding. Avidity is often expressed in terms of the ability of an antibody to effect a biological antigen-binding function. The distinction may be clarified by considering that an antibody of constant avidity can react with a large number of different, but related, antigens of different affinity.

In RIA, the sensitivity of an antiserum is primarily dependent upon its avidity (282). Standard RIA curves have therefore been constructed for cyclic GMP antibodies in order to compare sensitivity. All other factors which may influence sensitivity e.g. assay volume and equilibration time (212), have been kept constant. In the following discussion, therefore, sensitivity may be regarded as synonomous with avidity. The quantity of tracer has been selected to be as low as possible without introducing statistical counting errors due to too few counts. For comparison of standard curves, the dilution of each antibody (undiluted protein concentration adjusted to 20mg/ml) was selected which bound 30% of $[^{3}H]$ cyclic GMP (0.2 p moles per assay tube), in the absence of unlabelled cyclic GMP. Standard amounts of unlabelled cyclic GMP were then added to displace this 'zero binding' from ~6,000 counts/4 minutes

to ~1600 counts/4 minutes. Zero binding was converted to 100% to eliminate the slight variations around 30% binding by the different antibodies, and the relative percentage binding was calculated for each antibody with different amounts of unlabelled cyclic GMP. Graphs were then constructed of relative percentage binding of [^{3}H]cyclic GMP (ordinate) vs. amount of unlabelled cyclic GMP (abscissa, log scale). For example see figure 21, showing standard curves for 4 bleeds taken from rabbit A10. Samples were discarded if duplicate variation was greater than 5%. A zero binding level of 30% tracer was chosen to reduce the amounts of low titre antibody required for binding. An added advantage of low zero binding compared with (say) a 50% level, is that sensitivity or detection limit is increased; cross-reactivity to related antigens may also be decreased with a lower zero binding level (293). Reduction to very low levels however, limits the precision of the standard curve since the gradient is reduced (212).

A number of techniques have been used for comparing the sensitivity of antisera during immunization, from RIA standard curves (referred to in reference 282) e.g:

i) by comparison of the detection limits from the standard curves i.e. the amount of unlabelled antigen causing a statistical reduction in zero binding,

ii) by comparing the amount of unlabelled antigen required to reduce zero binding by different percentages,

iii) by reduction in zero binding of different antisera, using an arbitrarily selected quantity of unlabelled antigen.

Using the latter method Hurn and Landon, for example, compared the sensitivity of 24 antisera to insulin, and showed variation in

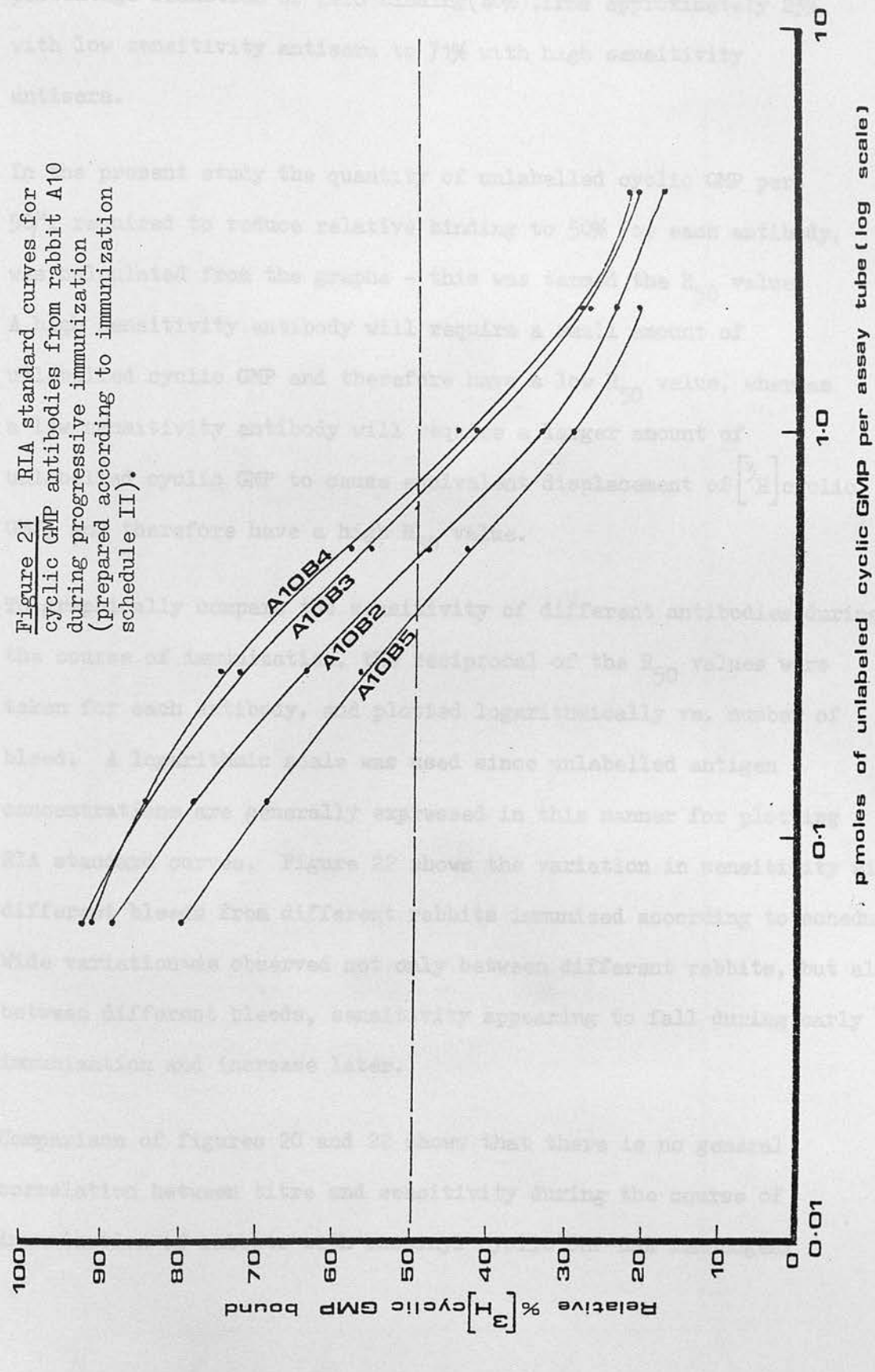


Figure 21 RIA standard curves for cyclic GMP antibodies from rabbit A10 during progressive immunization (prepared according to immunization schedule II).

percentage reduction of zero binding(40%),from approximately 23% with low sensitivity antisera to 71% with high sensitivity antisera.

In the present study the quantity of unlabelled cyclic GMP per 50μl required to reduce relative binding to 50% for each antibody, was calculated from the graphs - this was termed the R_{50} value. A high sensitivity antibody will require a small amount of unlabelled cyclic GMP and therefore have a low R_{50} value, whereas a low sensitivity antibody will require a larger amount of unlabelled cyclic GMP to cause equivalent displacement of $[^3H]$cyclic GMP, and therefore have a high R_{50} value.

To graphically compare the sensitivity of different antibodies during the course of immunization, the reciprocal of the R_{50} values were taken for each antibody, and plotted logarithmically vs. number of bleed. A logarithmic scale was used since unlabelled antigen concentrations are generally expressed in this manner for plotting RIA standard curves. Figure 22 shows the variation in sensitivity with different bleeds from different rabbits immunized according to schedule II. Wide variationwas observed not only between different rabbits, but also between different bleeds, sensitivity appearing to fall during early immunization and increase later.

Comparison of figures 20 and 22 shows that there is no general correlation between titre and sensitivity during the course of immunization of rabbits with succinyl cyclic GMP-HSA immunogen.

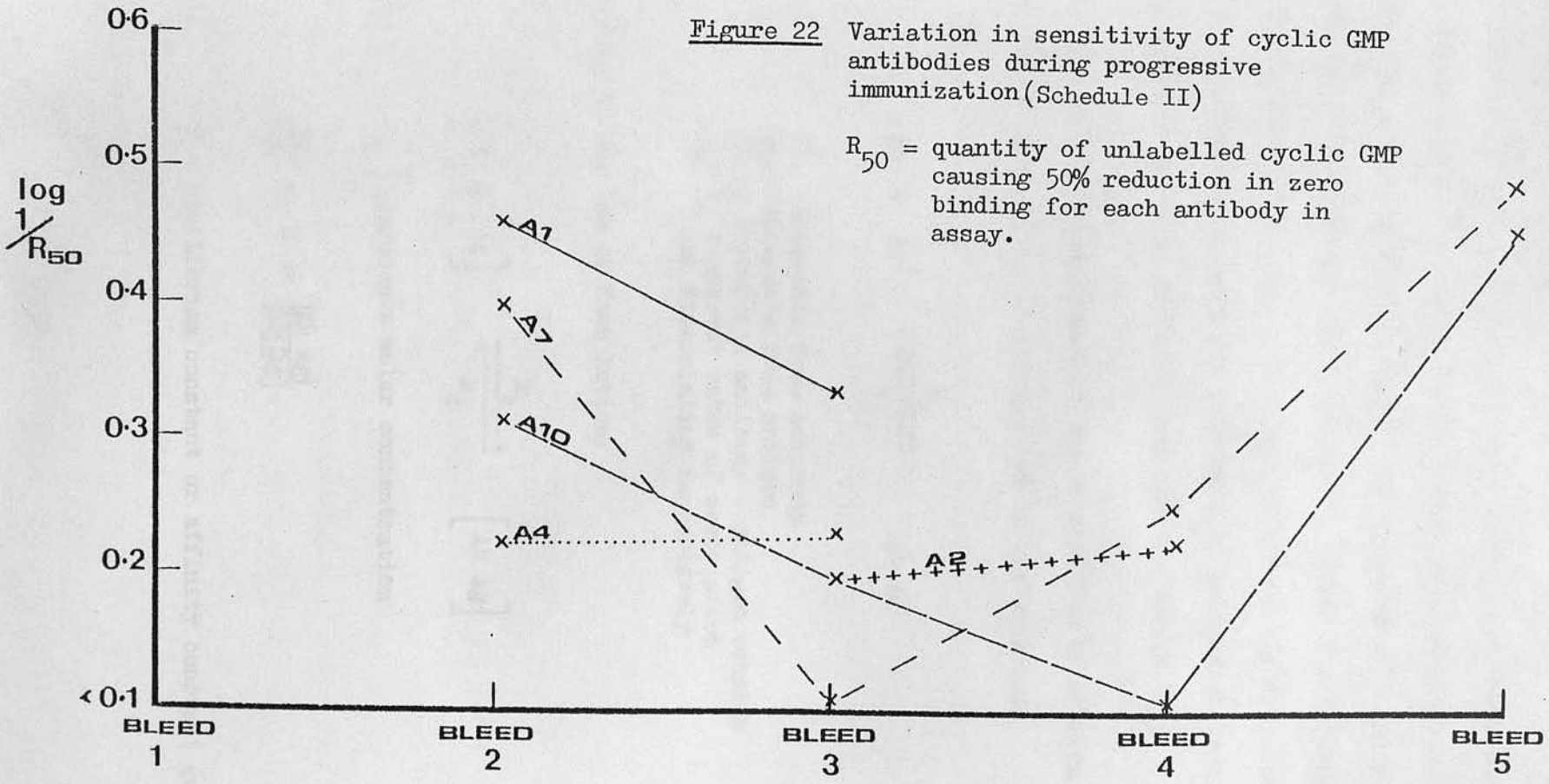


Figure 22 Variation in sensitivity of cyclic GMP antibodies during progressive immunization (Schedule II)

R_{50} = quantity of unlabelled cyclic GMP causing 50% reduction in zero binding for each antibody in assay.

4.5. Measurement of affinity constants and indices of affinity heterogeneity, of selected cyclic GMP antibodies.

i) Derivation of formulae.

The previous section compared the sensitivity, and therefore avidity, of a large number of cyclic GMP antibodies obtained from rabbits during the course of immunization. For calculation of affinity constants of antibodies, the technique of Nisonoff and Pressman (294) was used, enabling calculation of not only mean affinity constants, but also deviation of affinity constants of antibodies from this value - heterogeneity index of affinity (see also reference 193).

The quantitative relationship of the interaction of antigen and antibody at equilibrium is represented by the equation:

$$Ab + Ag \underset{k_d}{\overset{k_a}{\rightleftharpoons}} Ab\,Ag$$

where: Ab, represents free antibody
Ag, represents free antigen
Ab Ag, represents antibody - antigen complex
k_a, k_d represent rates of association and dissociation respectively

According to the Law of Mass Action:

$$[Ab] + [Ag] \underset{k_d}{\overset{k_a}{\rightleftharpoons}} [Ab\,Ag]$$

where: $[\ ]$ represents molar concentration

$$\frac{ka}{kd} = K = \frac{[Ab\,Ag]}{[Ab][Ag]}$$

where: K = equilibrium constant or affinity constant (units, M^{-1})

From the Law of Mass Action, the Langmuir absorption isotherm may be derived:

$$\frac{[Ab\ Ag]}{[Ab]} = \frac{nK[Ag]}{1+K[Ag]}$$

where: n = number of antbody binding sites, valency

This may be alternatively expressed as:

$$\frac{b}{n[Ab]} = \frac{K[Ag]}{1+K[Ag]}$$

where: b = bound antigen concentration $[Ab\ Ag]$

If isolation and purification of the antibody are not possible for determining antibody concentration, K may be calculated from this equation with respect to total antibody binding sites $[Ab_t]$, rather than $[Ab]$ and n. The equation may therefore be expressed as:

$$\frac{b}{[Ab_t]} = \frac{K[Ag]}{1+K[Ag]}$$

$$\therefore \frac{1}{b} = \left[\frac{1}{[Ab_t]} \cdot \frac{1}{K} \cdot \frac{1}{[Ag]}\right] + \frac{1}{Ab_t}$$

$[Ab_t]$ may be determined experimentally by extrapolation of the straight line plot resulting from $\frac{1}{b}$ vs $\frac{1}{[Ag]}$, since when:

$$\frac{1}{[Ag]} = 0, \quad \frac{1}{b} = \frac{1}{[Ab_t]}$$

Due to the heterogeneity of antibody affinity however, the theoretical straight line plot may deviate from linearity. The value of K is therefore calculated from a plot of the logarithmic transformation of the Sips equation (see reference 295):

$$\frac{r}{n} = \frac{K[Ag]^a}{1+K[Ag]^a}$$

Thus:

$$\log \frac{r}{n-r} = a \log K + a \log [Ag] \quad ***$$

where: r = moles of antigen bound per mole of antibody
a = heterogeneity index of affinity

Since the valency of IgG is 2,

$$[Ab] = \frac{Ab_t}{2} \qquad \text{since } [Ab_t] = n[Ab]$$

$$\therefore \quad r = \frac{b}{Ab_t / 2}$$

$$\therefore \quad \log \frac{r}{n-r} = \log \left[\frac{\frac{b}{Ab_t/2}}{2 - \frac{b}{Ab_t/2}} \right]$$

$$= \log \frac{b}{[Ab_t] - b}$$

Therefore in a plot of $\log \frac{b}{Ab_t - b}$ vs. $\log [Ag]$ (see***),

when: $\log \frac{b}{Ab_t - b} = 0, \quad K = \frac{1}{[Ag]}$

The heterogeneity index, a, is given by the slope of this plot. As the slope approaches 1, the antibody population approaches homogeneity with respect to association constants.

ii) Experimental procedure - determination of $[Ab_t]$, a and K.

Four antibodies showing different results by immunofluorescence, were examined in detail. Dilutions of antibodies binding approximately 30% of tracer in the binding assay were incubated under the same conditions, but with varying quantities of $[^3H]$cyclic GMP, from 0.08 - 0.53 p moles per assay tube. Calculation of percentage tracer bound at each concentration, enabled the quantity of $[^3H]$cyclic GMP bound and free, to be calculated in p moles. The amount of $[^3H]$ cyclic GMP bound by non-immune serum was calculated for each concentration of tracer and subtracted. All experimental determinations were carried out in duplicate. The data is summarised in table 6.

$[Ab_t]$ was calculated from the plots of $\frac{1}{b}$ vs $\frac{1}{f}$ as the intercept on the ordinate, when $\frac{1}{f} = 0$. $[Ab_t]$ values for each antibody were subsequently used for calculation of a and K from the plot of

$$\log \frac{b}{[Ab_t] - b} \text{ vs. } \log f.$$

Results shown in table 7 were calculated from lines of 'best fit', using an Olivetti programmable calculator.

Since values are generally expressed as dissociation constants, K_d values were calculated for the cyclic GMP antibodies, and ranged from 2×10^{-10} to 2×10^{-9}M.

K_d values of between 1.5 and 4.1×10^{-9}M have been found by a similar method for cyclic GMP antisera (296) and Wood and Marks (297) have also calculated K_d values for three cyclic GMP antisera of 3.1×10^{-9}M, 1×10^{-9}M and 1.8×10^{-8}M.

The heterogeneity index of the four cyclic GMP antibodies examined was close to 1.0, indicating that the population of antibody molecules were relatively homogeneous with respect to affinity.

Table 6 Data used for calculation of mean dissociation constants and heterogeneity indices of affinity for selected cyclic GMP antibodies.

		p moles of [^{3}H] cGMP in assay tube	p moles of [^{3}H] cGMP bound (non-specific binding subtracted) b	$1/b$	p moles of [^{3}H] cGMP free f	$1/f$	Ab_t – total binding sites (from graph) $\frac{b}{Ab_t - b}$	log converted to negative number $\log \frac{b}{Ab_t - b}$	log converted to negative number log f
A10B5	antibody	0.08	0.027	37.0	0.053	18.9	0.27	-0.5686	-1.2757
		0.16	0.048	20.8	0.112	8.9	0.60	-0.2218	-0.9508
		0.27	0.065	15.4	0.205	4.9	1.03	0.0128	-0.6882
		0.40	0.080	12.5	0.320	3.1	1.67	0.2227	-0.4949
		0.53	0.085	11.8	0.445	2.2	1.98	0.2967	-0.3516
A7B2	antibody	0.08	0.027	37.0	0.053	18.9	0.128	-0.8928	-1.2757
		0.16	0.050	20.0	0.110	9.1	0.266	-0.5751	-0.9586
		0.27	0.076	13.2	0.194	5.2	0.469	-0.3288	-0.7122
		0.40	0.098	10.2	0.302	3.3	0.700	-0.1549	-0.5200
		0.53	0.115	8.7	0.415	2.4	0.935	-0.0292	-0.3820
AMERSHAM	antibody	0.08	0.035	28.6	0.045	22.2	0.255	-0.594	-1.3468
		0.16	0.061	16.4	0.099	10.1	0.550	-0.2596	-1.0044
		0.27	0.086	11.6	0.184	5.4	1.000	0	-0.7352
		0.40	0.105	9.5	0.295	3.4	1.570	0.1959	-0.5302
		0.53	0.111	9.0	0.419	2.4	1.820	0.2601	-0.3778
STEINER G7 7/15/74	antibody	0.08	0.029	34.5	0.051	19.6	0.186	-0.7305	-1.2924
		0.16	0.052	19.2	0.108	9.3	0.391	-0.4078	-0.9666
		0.4	0.092	10.9	0.308	3.2	0.989	-0.0048	-0.5114
STEINER G28	antibody	0.08	0.042	23.8	0.038	26.3	0.913	-0.0395	-1.4202
		0.114	0.053	18.9	0.061	16.4	1.514	0.1797	-1.2147
		0.16	0.058	17.2	0.102	9.8	1.933	0.2863	-0.9914
		0.27	0.070	14.3	0.200	5.0	3.889	0.5897	-0.6990
		0.4	0.078	12.8	0.322	3.1	7.800	0.8921	-0.4949

Table 7 Mean dissociation constants and heterogeneity indices of affinity for selected cyclic GMP antibodies.

CYCLIC GMP ANTIBODY	HETEROGENEITY INDEX a	CALCULATED Kd	Kd
A10 B5	1.0	0.21p mole/200µl	1.1×10^{-9}M
A7 B2	1.0	0.40p mole/200µl	2.0×10^{-9}M
AMERSHAM	1.0	0.19p mole/200µl	1.0×10^{-9}M
STEINER G17 7/15/74	1.0	0.30p mole/200µl	1.5×10^{-9}M
STEINER G28	0.9	0.042p mole/200µl	2.0×10^{-10}M

Total assay volume = 200µl

4.6. Measurement of cross-reactivity of selected cyclic GMP antibodies with closely related nucleotides.

4.6.A. Determination of complete cross-reactivity data for selected cyclic GMP antibodies.

Cyclic GMP and related nucleotides were compared in a RIA system for their capacity to displace $[^3H]$ cyclic GMP from three antibodies, which showed different results by immunofluorescence. An antibody with high specificity for cyclic GMP would be expected to require large amounts of other nucleotides to compete with, and displace $[^3H]$ cyclic GMP; a low specificity antibody however, being unable to clearly distinguish cyclic GMP from related nucleotides, would only require small amounts of these compounds to displace an equivalent quantity of $[^3H]$ cyclic GMP. It was hoped that examination of cross-reactivity data would help to determine the sites, or determinants, on the cyclic GMP molecule, conferring specificity of binding.

The cyclic GMP antibodies were tested against cyclic GMP and 14 related purine, and one pyrimidine, nucleotides (Sigma Chemical Co.). Standard cyclic GMP RIA curves were constructed for each antibody as previously described, with 0.2p moles of $[^3H]$ cyclic GMP, a zero binding level of 30%, and 10^{-7} - 10^{-15} moles of related nucleotide in a volume of 50μl, in place of unlabelled cyclic GMP. Figure 23 shows cross-reactivity curves for A10 B5 antibody.

A cross-reactivity index was calculated for each nucleotide, as the ratio of the amount of nucleotide to the amount of cyclic GMP, required to reduce relative binding to 50% (data shown in table 8; see also reference 231).

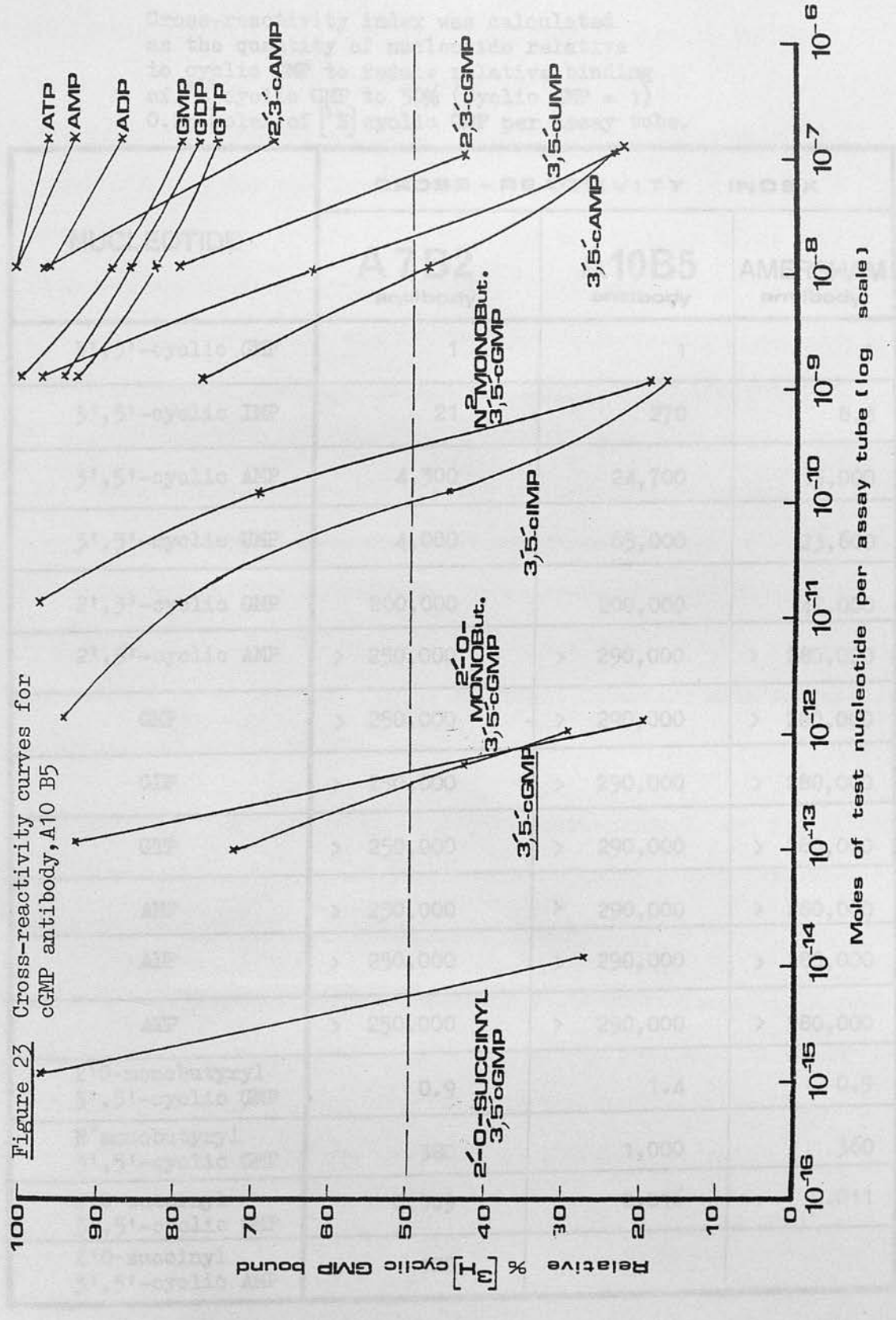


Figure 23 Cross-reactivity curves for cGMP antibody, A10 B5
Relative % [125I] cyclic GMP bound
100
90
80
70
60
50
40
30
20
10
0
Moles of test nucleotide per assay tube (log scale)
10-16
10-15
10-14
10-13
10-12
10-11
10-10
10-9
10-8
10-7
10-6
ATP
AMP
ADP
GMP
GDP
GTP
2',3'-cAMP
2',3'-cGMP
3',5'-cUMP
3',5'-cAMP
N2-MONOBut. 3',5'-cGMP
3',5'-cIMP
2'-O-MONOBut. 3',5'-cGMP
3',5'-cGMP
2'-O-SUCCINYL 3',5'-cGMP

Table 8 Cross-reactivity of selected cyclic GMP antibodies to related nucleotides.

Cross-reactivity index was calculated as the quantity of nucleotide relative to cyclic GMP to reduce relative binding of [^{3}H] cyclic GMP to 50% (cyclic GMP = 1) 0.2p moles of [^{3}H] cyclic GMP per assay tube.

NUCLEOTIDE	CROSS - REACTIVITY INDEX		
	A7B2 antibody	A10B5 antibody	AMERSHAM antibody
3',5'-cyclic GMP	1	1	1
3',5'-cyclic IMP	21	270	8.3
3',5'-cyclic AMP	4,300	24,700	15,000
3',5'-cyclic UMP	4,000	65,000	23,600
2',3'-cyclic GMP	200,000	200,000	47,000
2',3'-cyclic AMP	> 250,000	> 290,000	> 280,000
GMP	> 250,000	> 290,000	> 280,000
GDP	> 250,000	> 290,000	> 280,000
GTP	> 250,000	> 290,000	> 280,000
AMP	> 250,000	> 290,000	> 280,000
ADP	> 250,000	> 290,000	> 280,000
ATP	> 250,000	> 290,000	> 280,000
2'0-monobutyryl 3',5'-cyclic GMP	0.9	1.4	0.9
N^2monobutyryl 3',5'-cyclic GMP	380	1,000	360
2'0-succinyl 3',5'-cyclic GMP	0.009	0.016	0.011
2'0-succinyl 3',5'-cyclic AMP	100		

4.6.B. Discussion

i) The three antibodies showed similar cross-reactivity, although A10 B5 was slightly more specific. The data is similar to that reported for other cyclic GMP antibodies (231, 297).

ii) Synthetic succinyl cyclic GMP showed approximately 100-fold higher affinity for the three antibodies, compared with the naturally occurring cyclic GMP. This may be explained since the immunogen was succinyl cyclic GMP-HSA. This increased affinity has been exploited by Cailla et al (233, 234) using succinylated samples and standards, to increase the sensitivity of cyclic nucleotide RIA to the femtomolar level (see also page 305 for acetylation of cyclic nucleotides)

iii) A prime determinant for specificity appeared to be the 3',5' phosphodiester bond, since adenosine and guanosine mono, di, and tri-phosphate nucleotides showed very low cross-reactivity ($> 250,000$). This is of particular value since ATP, for example, occurs at high concentrations in the cell (231), and might interfere with measurement of cyclic GMP levels by RIA.

Even when the phosphate group is linked between the 2' and 3' positions on the ribose moiety (cyclic 2',3'-monophosphates), cross-reactivity was very low, stressing the importance of the 3',5' determinant.

It is interesting that whilst 3',5' and 2',3' derivatives all showed parallel RIA curves suggesting competition at the same site on the cyclic GMP antibody, this was not observed with non-cyclic nucleotides, suggesting that binding was occurring at different sites.

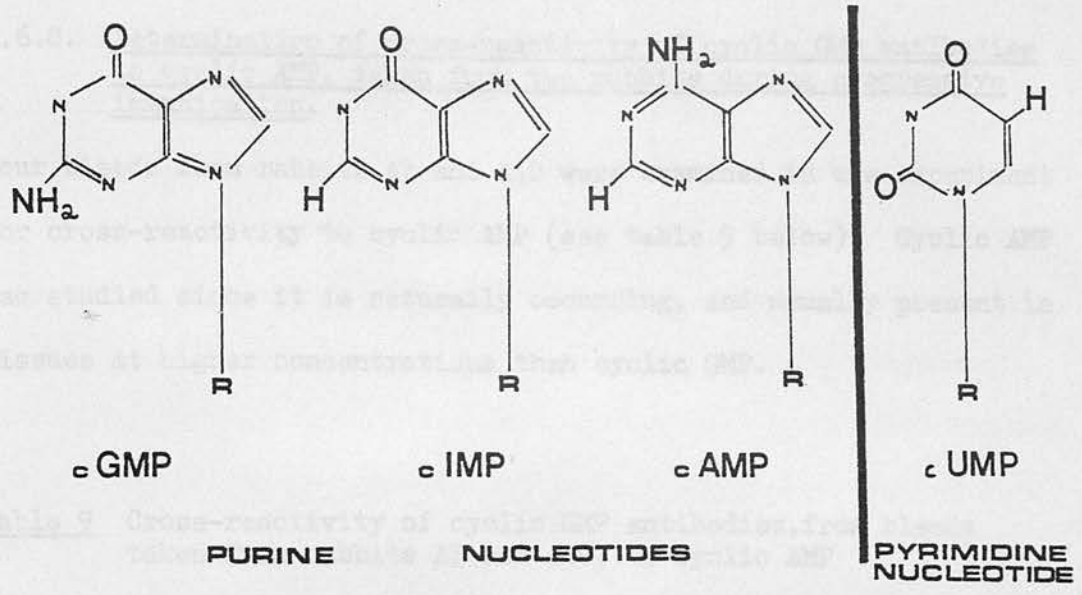


Figure 24 Comparison of structures of cyclic nucleotides.

Cyclic IMP cross-reacted with cyclic GMP to the greatest extent in comparison with the other cyclic nucleotides tested. Examination of the structures of the cyclic nucleotides (see figure 24 above) explains these observations, on the basis of molecular similarities.

The importance of the 3',5' group in determining specificity relative to nucleotides without this cyclic structure, may be related to the 3',5' bond altering the plane of the ribose moiety in cyclic nucleotides (as demonstrated in reference 298).

Substitution of a butyryl group on the 2'-position of the ribose sugar, had very little effect on affinity relative to cyclic GMP, whilst substitution of this uncharged group on the N^2 position on the purine moiety, caused a modest decrease in affinity.

These studies show that a prime antigenic determinant on the cyclic GMP molecule is the 3',5' cyclic structure, with substituents on the purine moiety conferring additional specificity.

4.6.C. Determination of cross-reactivity of cyclic GMP antibodies to cyclic AMP, taken from two rabbits during progressive immunization.

Four bleeds from rabbits A7 and A10 were examined in one experiment for cross-reactivity to cyclic AMP (see table 9 below). Cyclic AMP was studied since it is naturally occurring, and usually present in tissues at higher concentrations than cyclic GMP.

Table 9 Cross-reactivity of cyclic GMP antibodies, from bleeds taken from rabbits A7 and A10, to cyclic AMP

ANTIBODY	CROSS-REACTIVITY INDEX
A7 BLEED 2	4,000
A7 BLEED 3	2,125
A7 BLEED 4	6,786
A7 BLEED 5	11,250
A10 BLEED 2	27,660
A10 BLEED 3	11,406
A10 BLEED 4	9,066
A10 BLEED 5	23,235

Comparison of the data in table 9 with that in figure 22, shows a correlation in sensitivity and cross-reactivity (to cyclic AMP), during the course of immunization. This correlation is to be expected (250), since a high avidity antibody to cyclic GMP with binding sites 'tailored' to cyclic GMP, would not be expected to recognize and bind cyclic AMP as effectively.

5. COMPARISON OF CHARACTERISTICS OF CYCLIC GMP ANTIBODIES AS MEASURED USING RADIOIMMUNOLOGICAL TECHNIQUES, WITH ABILITY TO SHOW POSITIVE IMMUNOFLUORESCENT STAINING.

To determine whether the binding of antibodies to nucleotide in a RIA system could be used to predict their usefulness in detecting tissue-bound nucleotide for immunofluorescence studies, cyclic GMP antibodies from immunization schedule II, Dr Steiner's laboratory and the Radiochemical Centre, were ranked according to their binding characteristics (titre, avidity and specificity), and compared with results by immunofluorescence.

5.1. Immunofluorescence ranking of cyclic GMP antibodies.

6μm rat cerebellar sections were incubated overnight, in an attempt to maximize specific staining (see page 185)with a range of dilutions of cyclic GMP antibodies, and equivalent concentrations of immunoglobulin from unimmunized rabbits. It was found that whilst the majority showed no difference in staining in comparison with non-immune immunoglobulins, and were therefore classed as negative, positive staining antibodies could be classified as localizing FIBRES or CAPILLARIES in cerebellar sections (for example, see figures 39, 49, and 50). The characterization of the positive staining structures is discussed in chapter IV. Three bleeds tested from one of Dr Steiner's rabbits, G17 (raised against succinyl cyclic GMP-KLH immunogen), showed particularly bright fibre staining, but surprisingly,whilst the three bleeds all specifically localized this component, one bleed in addition, showed capillary staining.(Rabbit G17 was boosted with immunogen between each bleed):

STEINER	G17	7/15/74	JULY 1974 BLEED	——	FIBRE STAINING (e.g.Fig 49)
STEINER	G17	10/31/74	OCT 1974 BLEED	——	FIBRE STAINING
STEINER	G17	12/2/74	DEC 1974 BLEED	——	FIBRE AND CAPILLARY STAINING (e.g.Fig 50)

Positive staining antibodies were ranked on the basis of intensity of specific staining relative to 'background' (see page 188). Non-specific staining of cerebellar tissue sections could be clearly observed with concentrations of non-immune immunoglobulin (prepared by ammonium sulphate fractionation) in excess of approximately 1mg protein/ml, the intensity increasing with concentration.

The positive staining described in this thesis with cyclic AMP and cyclic GMP antibodies: G17, A10 B5, G28, #7 and TEX cyclic AMP, was in the range of 0.2 - 0.8mg protein/ml; A4 B2 and A7 B2 cyclic GMP antibodies were employed at 1mg protein/ml. With these latter antibodies, brightly staining fibres could be clearly distinguished against the background non-specific staining of the Purkinje cells and structures in the granule layer, which probably represent the glomeruli (see figure 25 and also figures 53 and 58).

Specific cyclic nucleotide antibodies were screened under identical conditions to non-immune immunoglobulin from the same animal, at the same concentration, for each brain area and tissue studied. It was often helpful to also employ higher concentrations of non-immune immunoglobulin, in order to maximize non-specific staining structures, as suggested by Hartman (257).

5.2. Comparison of ranking of cyclic GMP antibodies using radioimmunological and immunofluorescence techniques.

Examination of table 10 demonstrates that there is no binding characteristic(s) that either distinguishes positive or negative staining antibodies on immunofluorescence, or predicts immunofluorescent ranking. Furthermore, the extensive cross-reactivity studies carried out in section 4.6, were unable to show any significant differences

Figure 25 Immunofluorescent localization of cyclic GMP in rat cerebellum using antibodies A7 B2, Steiner G28 and non-immune immunoglobulin.

a) A7 B2 antibody
b) Steiner G28 antibody
c) Non-immune immunoglobulin

Calibration bar = 50µm

P - Purkinje cell body

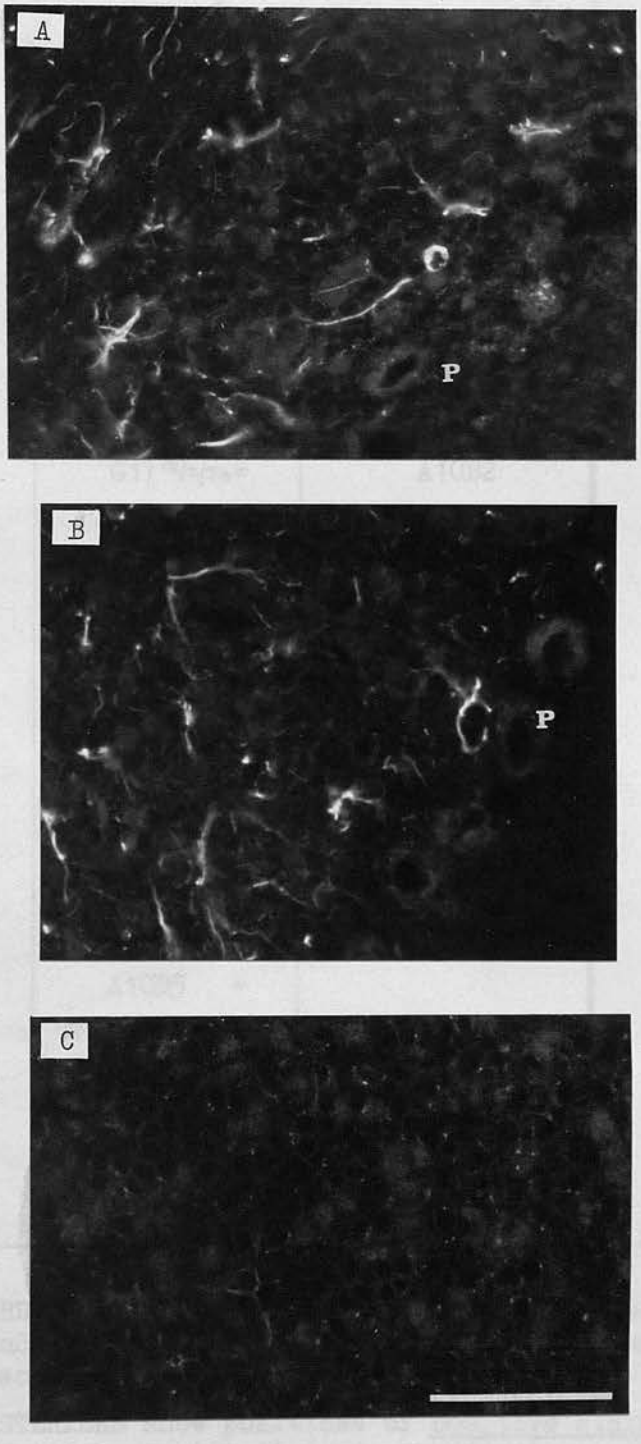


Table 10 Ranking of cyclic GMP antibodies according to titre, sensitivity and cross-reactivity, and ability to show positive immunofluorescence in rat cerebellar sections.

TITRE	SENSITIVITY	CROSS-REACTIVITY TO CYCLIC AMP	FIBRE STAINING	CAPILLARY STAINING
A10B3 ↓	A7B3 ↓	A7B3 ↓	A4B2 ↓	G17 12/2/74
A9B4	A10B4	A7B2 *	A7B2	A10B5
A7B2 *	A2B3	A7B4	G28	
A2B3	A10B3	A10B4	G17 12/2/74	
A10B5 *	A2B4	A7B5	G17 7/15/74	
A7B3	A4B2 *	A10B3		
A4B2 *	A4B3	A10B5 *		
A10B2	G17 12/2/74 *	A10B2		
A1B3	A7B4			
A2B4	A10B2			
A7B4	A1B3			
G17 7/15/74 *	A7B2 *			
A1B2	G17 7/15/74 *			
A10B4	AMERSHAM			
A4B3	A1B2			
G17 12/2/74 *	A10B5 *			
A7B5	A7B5			
G28 *	G28 *			
AMERSHAM				

NOTE: i) ARROWS show direction of increasing titre, sensitivity, specificity and intensity of specific immunofluorescent staining (relative to background).

ii) ASTERISKS show positions of positive staining antibodies in ranking of titre, sensitivity and specificity.

between a fibre staining antibody (A7 B2), a capillary staining antibody (A10 B5), and one that is negative by immunofluorescence (Amersham). These three antibodies together with Steiner G17 12/2/74 and G28, were also shown to be homogeneous with respect to affinity constants; this demonstrates that the positive antibodies do not contain, for example, a small population of molecules with very high affinity for cyclic GMP in the assay, which might be responsible for the positive staining patterns.

5.3. To determine whether cyclic GMP antibodies might have binding sites masked by endogenous nucleotide.

Since both cyclic AMP and cyclic GMP are found circulating in the plasma of unimmunized animals (299), it was possible that production of antibodies in the plasma on repeated immunization could bind the circulating nucleotides, thereby masking some,or all,of the cyclic nucleotide antibody binding sites. In the case of low avidity antibodies, bound nucleotide is probably removed when the serum is fractioned with ammonium sulphate and the immunoglobulin fraction is extensively dialyzed. For the high avidity population, however, this procedure may not be sufficient to unmask all the binding sites. There exists the possibility, therefore, that the reason for the small number of antibodies showing positive immunofluorescence is due to this 'masking' effect. Steiner et al (215) have demonstrated that endogenous cyclic nucleotides can be removed from antibody under mild conditions which cleave the antibody-antigen bond, exposing binding sites which may prove useful in immunofluorescence. Following their methodology, three antibodies and a non-immune control were selected,and dialyzed with constant stirring (see page121) vs. 0.1M glycine-HCl buffer at pH 2.2,overnight at 4°C, followed by dialysis vs. PBS for an additional 3 days at 4°C,

with three changes of buffer. The antibodies were then centrifuged at 15,000g for 15 minutes at 4°C (Burkard 'Koolspin' centrifuge), to remove the slight cloudiness which resulted from this procedure. 'Untreated'antibodies were compared with'treated'antibodies, using overnight incubation periods on frozen sections of rat cerebellum. The three antibodies were originally selected since one demonstrated fibre staining (A7 B2), one capillary staining (A10 B5), and one was negative on immunofluorescence (Amersham). After treatment there was no change, however, in these staining patterns, although there was increased non-specific fluorescence with the immune and non-immune immunoglobulin fractions. Similar effects were observed by Steiner et al (215), with certain antibodies, and may be attributed to irreversible damage caused to the immunoglobulin during the denaturation and renaturation dialysis steps.

6. GENERAL DISCUSSION

The characteristics of a large number of cyclic GMP antibodies have been examined using radioimmunological methods, in order to select antibodies for immunofluorescence on the basis of their titre, avidity or specificity. If the successful characteristic could be found, screening of antibodies for immunohistochemistry would be rapid, and specific animals might be selected for a continual immunization programme. The development of these three antibody characteristics was monitored during progressive immunization of 10 rabbits with succinyl cyclic GMP-HSA immunogen. The results showed that not only do individual rabbits show varying immunological responses, but also that wide variation occurs between different bleeds.

The results also showed that antibodies demonstrating positive immunofluorescent staining, could not be distinguished by the binding characteristics studied. Since slightly different staining patterns were observed with the positive immunohistochemical antibodies for cyclic GMP, a further object of the radioimmunological screening was to determine whether any of the binding characteristics were responsible. Again, neither titre, avidity nor specificity appeared to correlate with immunohistochemical staining patterns. Similar results were also obtained with cyclic GMP antibodies produced in Dr Steiner's laboratory and the Radiochemical Centre, which were tested in this study. The results suggest, therefore, that a larger pool of rabbits with selection using immunohistochemical rather than radioimmunological criteria, should be employed in future studies.

Although these experiments are, to my knowledge, the only detailed examination of radioimmunological techniques as potential screening methods for antibodies for use in immunohistochemistry, a number of isolated reports have shown similar results. Swaab and Pool (300) for example, have stated that during progressive immunization, the ability of an antiserum to show positive immunofluorescence, decreased to such a point that it could not be used, whereas the titre by RIA remained high. An antiserum of low avidity by RIA has been shown to produce good results for immunohistochemistry (217), whilst another report showed a high specificity antiserum by RIA to have low specificity for immunofluorescence (301).

To explain these discrepancies, the following differences between immunohistochemistry and RIA as techniques for detecting antigen should

be considered.

i) RIA detects antigen by competition with tracer for specific binding sites on the antibody. RIA will therefore only monitor those antibody molecules that bind tracer. This contrasts with immunohistochemistry, where all antibody molecules can bind and potentially localize their respective antigens, if present in the tissue section (302,303). The consequence of this difference is that the specificity controls differ for each procedure. By RIA the three cyclic GMP antibodies extensively examined for cross-reactivity with related nucleotides were highly specific. Specificity controls for immunohistochemistry are more rigorous, however, and require removal of the cyclic GMP antibody to correlate with removal of immunohistochemical staining (302, 303).

ii) RIA is an optimized, equilibrated assay system, which generally uses very high dilutions of primary antisera. In contrast, immunohistochemistry generally uses primary antibody at low dilution, for short incubation periods (303).

Discrepancies between equilibrium/non-equilibrium conditions in RIA and immunohistochemistry were eliminated in this study, however, using overnight incubation periods of tissue sections with cyclic GMP antibody. Also, similar quantities of cyclic GMP antibodies were employed per tissue section and per assay tube, probably due to the relatively low titre of cyclic nucleotide antibodies in a tritiated radioimmunological system.

iii) Perhaps the most important difference is that immunohistochemistry detects antigen which is bound in the tissue section causing its determinants to be modified or hidden, in contrast to RIA where the antigen is in solution with its determinants fully exposed. For example, it has been shown that antibodies generated against LH-RH conjugates 'see'

different portions of the molecule when bound in tissue sections for immunohistochemistry, whilst seeing the complete molecule in RIA (304).

Since an antiserum contains a population of antibody molecules with different avidities for antigen, perhaps the reason for only certain cyclic GMP antibodies showing positive immunofluorescence, is that these antibodies contain a population of molecules with a higher avidity for cyclic GMP bound in a tissue section, than free in solution. Different mechanisms of binding at different sites (as demonstrated for LH-RH, see reference 304) might also explain the different staining patterns with different cyclic GMP antibodies, as suggested by Steiner et al (215) and Dr E Rosenberg (personal communication).

This concept is discussed more fully in chapter 4.

CHAPTER IV

CHARACTERIZATION AND VALIDATION OF CYCLIC NUCLEOTIDE IMMUNOFLUORESCENT STAINING IN THE C.N.S.

CHAPTER IV

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5.2. To demonstrate that positive staining was not a result of antibodies raised against the carrier protein employed for immunization

5.3. To demonstrate that positive staining was due to cyclic GMP in the tissue section

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1. CYCLIC AMP IMMUNOHISTOCHEMISTRY

1.1. Rat cerebellum and brain stem

Employing antibodies Steiner#7 and Steiner#8 from rabbits immunized with succinyl cyclic AMP-KLH conjugate, positive staining was observed predominantly in the granule layer of the rat cerebellum, with minimal reactivity in the molecular layer and white matter (see figure 26). The diffuse granule layer staining was similar to that obtained with non-immune immunoglobulin, but could be clearly distinguished from non-specific staining on the basis of immunoglobulin concentration(see p.166).

To determine whether the post-mortem elevation of cyclic AMP could be localized by immunofluorescence, sections were processed identically with Steiner#7 antibody from rats whose cerebella were frozen within 30 seconds, or 2½ minutes after decapitation. Difficulty was found however, in removing the cerebella intact and immersing in the isopentane-liquid nitrogen freezing mixture within 30 seconds. Only after practise was it possible to freeze the tissue within this time, and this was inevitably accompanied by a certain amount of damage to the cerebellum. In confirmation of the work of Bloom et al (236), the post-mortem rise in cyclic AMP was localized as increased staining in the Purkinje cell bodies, with no significant change in the other cell types (see figure 26); cerebella from three rats frozen at each time interval, were used in this study.

Positive staining for cyclic AMP was also observed in the cytoplasm of selected large neurones in the brain stem, underlying the cerebellum. Positive staining neurones shown in figure 27 correspond to the mesencephalic trigemini nucleus, as judged by conventional histological localization of serially cut sections(toluidine blue staining, see appendix 4).

Figure 26 Immunofluorescent localization of cyclic AMP in rat cerebellum frozen in isopentane - liquid nitrogen within 30 seconds or 2½ mins after decapitation - antibody# 7.

a) 30 seconds after decapitation. Low magnification.
Calibration bar = 100 μm

b) 2½ minutes after decapitation. Low magnification.
Calibration bar = 100 μm

c) 2½ minutes after decapitation. High magnification.
Calibration bar = 50 μm

g - granule layer
m - molecular layer
P - Purkinje cell body layer

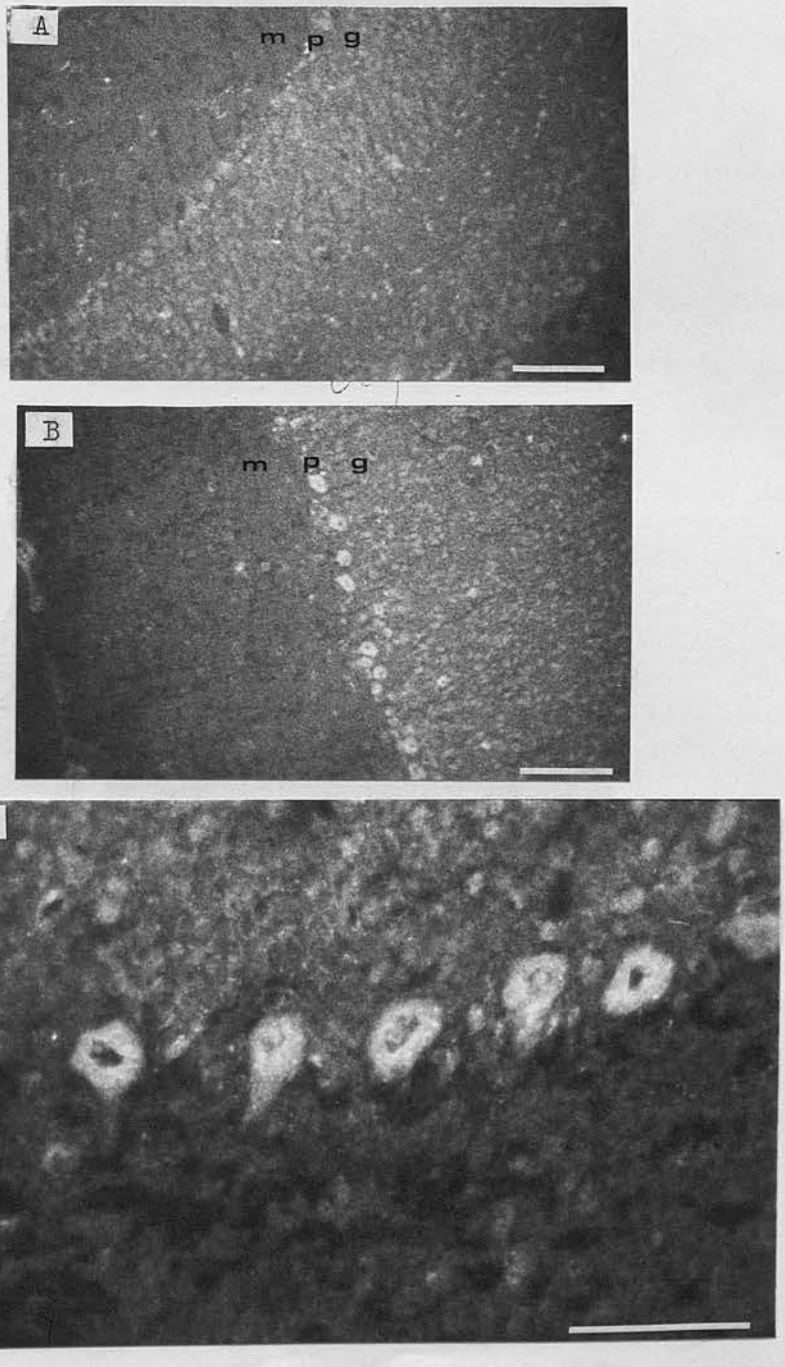


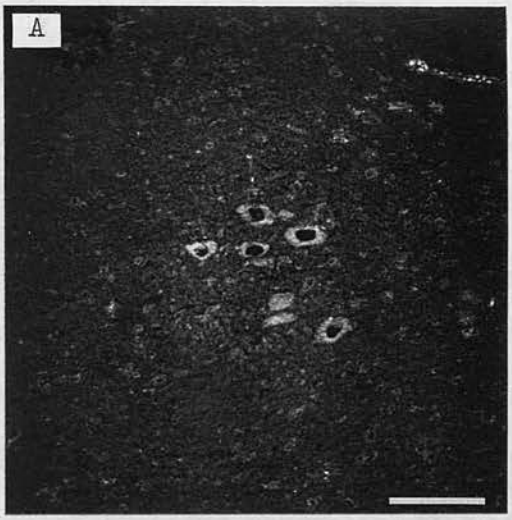
Figure 27 Comparative immunofluorescent localization of cyclic AMP and cyclic GMP in the brain stem of the rat.

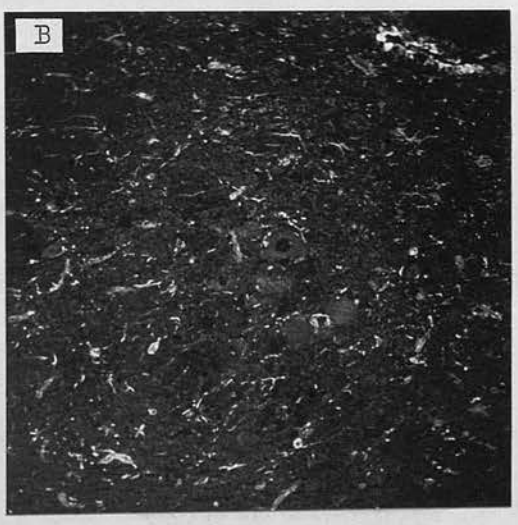
a) Cyclic AMP antibody#7

b) Cyclic GMP antibody, Steiner G17 12/2/74

} Calibration bar = 100µm

Antibodies used at the same immunoglobulin concentration. The large neurones prominent in a), and faintly visible in b), correspond to those of the mesencephalic trigemini nucleus





and reference to a neuroanatomical rat atlas (308).

Recently, I was able to examine a further cyclic AMP antibody for immunofluorescence supplied by Dr Steiner. The antibody, TEX cyclic AMP, was raised against a succinyl cyclic AMP-HSA conjugate, and had been shown to be highly specific by RIA, and to show immunofluorescence in rat liver sections with a distribution as reported previously (305). In rat cerebellum frozen 2½ minutes after decapitation (see figure 28), strong staining was observed in the granule layer, where the discrete localization to the granule cell nuclei clearly resembled that shown by Bloom's group (236), in contrast to the more diffuse staining shown by Steiner#7 antibody in this layer. (Note however, that in rabbit cerebellum, figure 43, diffuse staining in the granule layer was also found with TEX cyclic AMP antibody). Widespread staining was also observed with TEX cyclic AMP antibody in the molecular layer and white matter, the pattern of staining resembling that of the glial nuclei stained with toluidine blue. In further contrast to the report from Bloom's group (236), and the observations with Steiner#7 antibody, only occasional Purkinje cell fluorescence was observed in rat cerebellum, which was frozen 2½ minutes after decapitation.

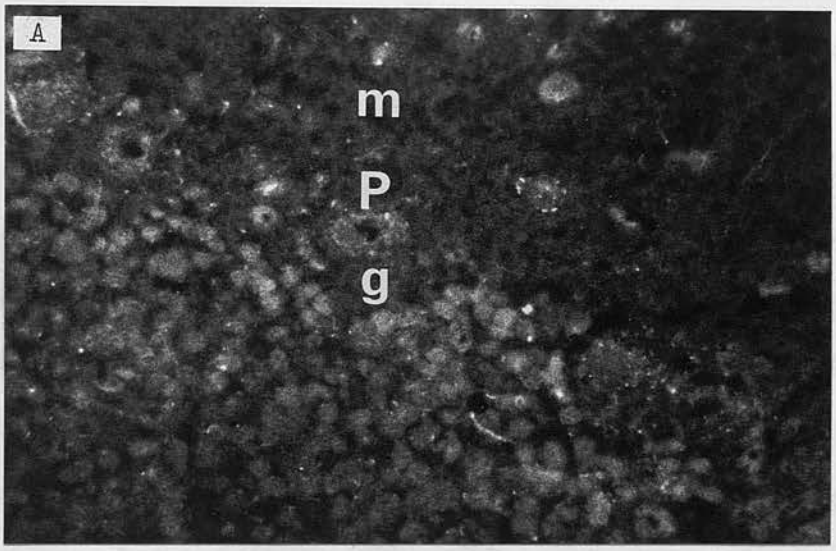
In the brain stem underlying the cerebellum, widespread staining of structures resembling the glial nuclei was observed with TEX cyclic AMP antibody, large neurones being unstained.

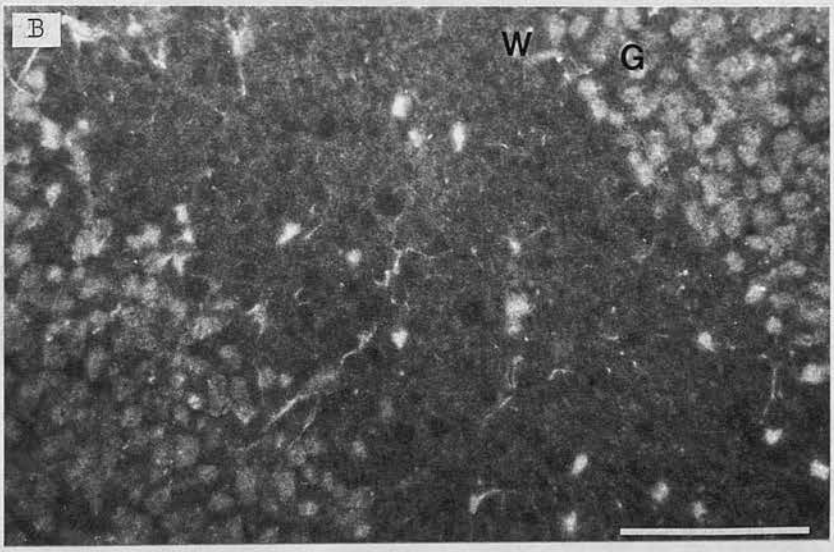
Distinction between neurones and glia might be studied using kainic acid, which selectively destroys neurones, whilst leaving glia intact and causing proliferation (309), prior to cyclic AMP immunofluorescence.

Figure 28 Immunofluorescent localization of cyclic AMP in rat cerebellum using TEX cyclic AMP antibody.

a) Granule and molecular layers
b) Granule layer and white matter } Calibration bar = 50 μm

g/G - granule layer
m - molecular layer
P - Purkinje cell body
W - white matter





1.2. Rat substantia nigra

Cyclic AMP immunofluorescence was performed with Steiner#7 and TEX cyclic AMP antibodies in the substantia nigra, a region of the brain known to contain neurotransmitter-sensitive adenylate cyclase (306).

Dissection of area B in figure 29, containing the substantia nigra, was performed from two rat brains, using a pair of long metal blades moistened with ice-cold physiological saline, as described in reference 307. The dissected wedge of tissue was placed on a cork mat and frozen in isopentane-liquid nitrogen (see page 86), approximately 5 minutes after decapitation. 6µm coronal sections were cut in the cryostat, and the substantia nigra was located using toluidine blue histology and reference to a neuroanatomical rat atlas (308); serial sections were then processed for cyclic AMP immunofluorescence. No staining was observed in the substantia nigra or surrounding areas with Steiner#7 antibody, whereas TEX cyclic AMP antibody revealed widespread staining resembling that of glial nuclei stained using toluidine blue; the large neurones in the substantia nigra were unstained (see figure 30).

1.3. Discussion

Extensive studies on the localization of cyclic AMP, have been severely limited by the availability of positive staining cyclic AMP antibodies. The studies reported here, however, have suggested that different antibodies may have different abilities for localizing cyclic AMP, perhaps as a result of slightly different binding characteristics at different sites; this theme has been pursued more vigorously for cyclic GMP in this thesis.

Figure 29 Diagram showing ventral aspect of the rat brain, and regions dissected for immunohistochemical experiments.

Region **A** was dissected for studies on the corpus striatum.

Region **B** was dissected for studies on the substantia nigra and choroid fissure.

Diagram adapted from (308).

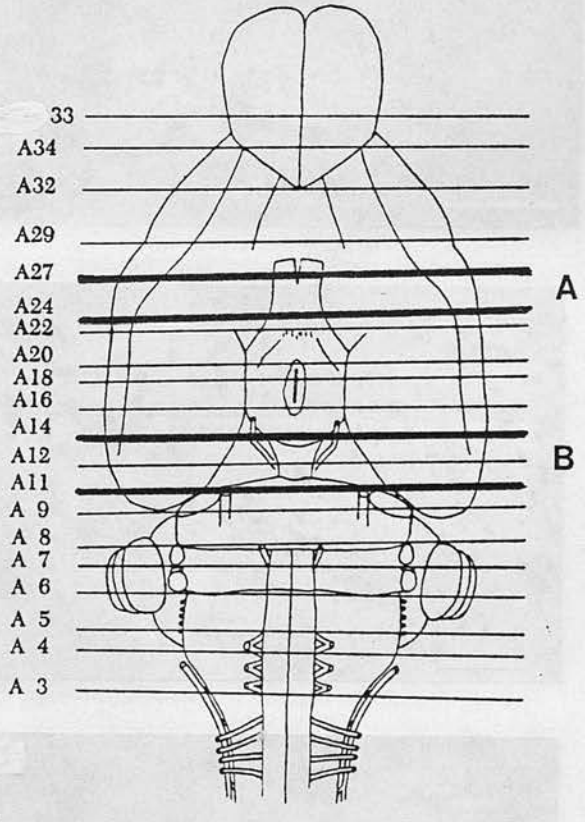


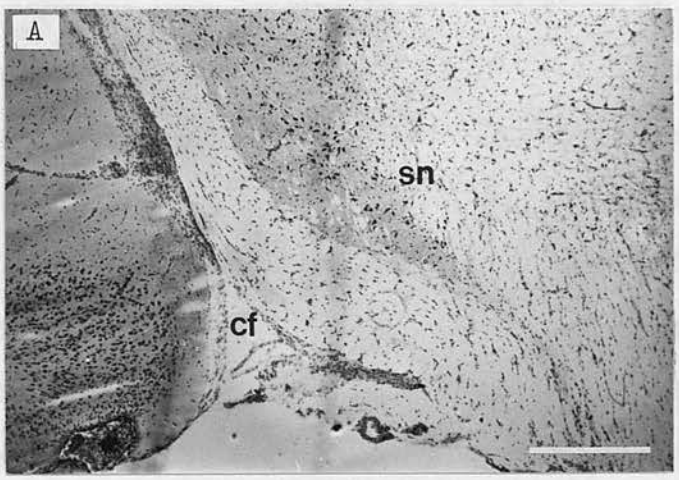
Figure 30 Immunofluorescent localization of cyclic AMP in rat substantia nigra using antibody TEX cyclic AMP.

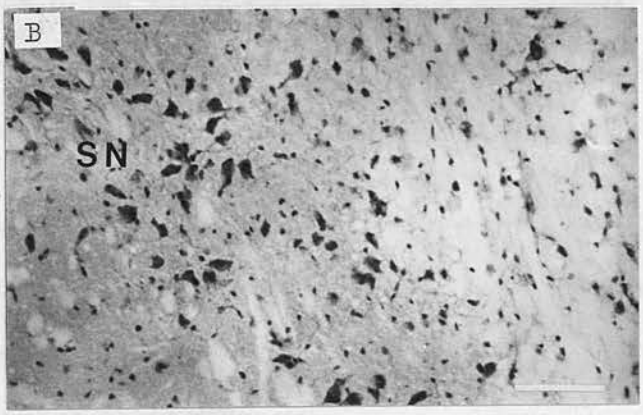
a) Toluidine blue staining to show substantia nigra and the choroid fissure. Low magnification. Calibration bar = 500 μm.

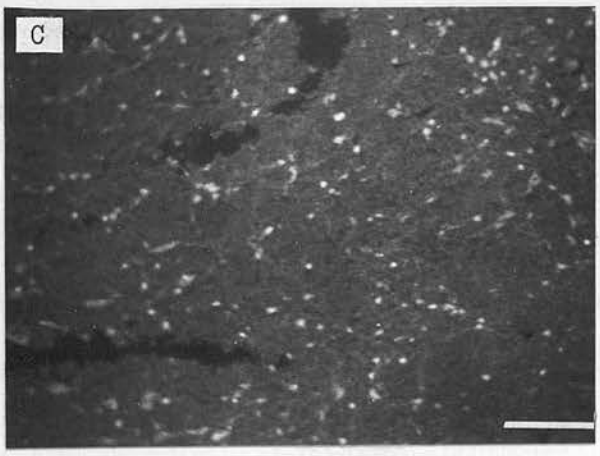
b) Toluidine blue staining to show the large neurones in the substantia nigra. High magnification. Calibration bar = 100 μm.

c) Cyclic AMP immunofluorescence in the region of the substantia nigra. High magnification. Calibration bar = 100 μm.

SN/sn - substantia nigra.
cf - choroid fissure.







2. CYCLIC GMP IMMUNOHISTOCHEMISTRY - OPTIMIZING CONDITIONS FOR POSITIVE IMMUNOFLUORESCENT STAINING

Certain aspects of the technique were examined, in order to maximize intensity of specific staining relative to 'background' immunofluorescence, using Steiner G17 cyclic GMP antibody.

2.1. Section thickness

10 - 14 μm sections were originally used by Bloom et al for immunofluorescent localization of cyclic AMP in cerebellum, whereas tissue sections for routine immunofluorescence are now cut in Dr Steiner's laboratory at 2 - 6 μm. The effect of section thickness (4 - 12 μm) on cyclic GMP immunofluorescence was examined. It was found that thinner sections produced clearer fibre staining than thick sections. The minimal thickness, however, is limited by the efficiency and temperature of the cryostat (275), and whilst 4 μm sections could be cut, difficulty was often experienced at this thickness. Since serial sectioning was an important requisite, section thickness was routinely set at 6 μm, to ensure successful sectioning.

Since immunofluorescence is dependent upon access of antibody to antigen in cells which have been cut open, reduction in section thickness would be expected to increase availability of antigen, and therefore improve staining.

2.2. Antibody incubation time and dilution

Modifications to the standard 30 minute incubation of first antibody e.g. increased temperature and increased incubation time, have been reported (318, 311). Positive immunofluorescence is dependent upon binding of specific antibody to tissue bound antigen. During a short 30 minute incubation period, we might expect all antibody

molecules to be available for binding to antigen, regardless of avidity, since the antigen-antibody interaction under these conditions, has probably not reached equilibrium. Increasing temperature and incubation time, however, will allow specific antigen-antibody interaction to reach equilibrium. Under these conditions, as for RIA, highest avidity antibody molecules will be bound to antigen.

Steiner et al (215) have found similar staining patterns of cyclic nucleotide immunofluorescence, using incubation times of first antibody, from minutes to many hours. These results suggest that the same molecules are involved for staining under non-equilibrium and equilibrium conditions. However, increasing incubation times allowed antibody dilutions to be markedly increased, reducing any non-specific effects and conserving valuable antibody. Experiments using Steiner G17 7/15/74 antibody incubated overnight (15 hours) on 6 µm rat cerebellar sections at room temperature, showed that antibody dilution could be increased 2 - 4 fold, and that whilst specific staining structures were the same as those observed at 30 minutes of incubation, non-specific background staining was reduced and specific staining was 'sharper' (see figure 31). The results may be interpreted as the reduction of non-specific staining being due to the decreased immunoglobulin concentration, and the 'sharper' specific fluorescence being due to increased amounts of cyclic GMP antibody bound, under equilibrium conditions.

Overnight incubation (15 hours) of first antibody was subsequently adopted, not only for cyclic GMP antibodies, but also for antibodies to other antigens. In all cases, extended incubation times showed clearer

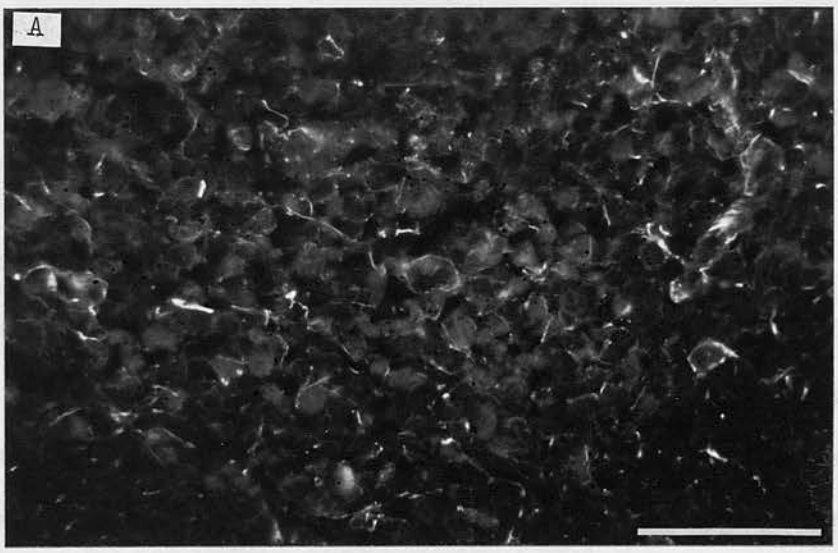
Figure 31 Immunofluorescent localization of cyclic GMP in rat cerebellum - comparison of incubation times and cyclic GMP antibody dilution.

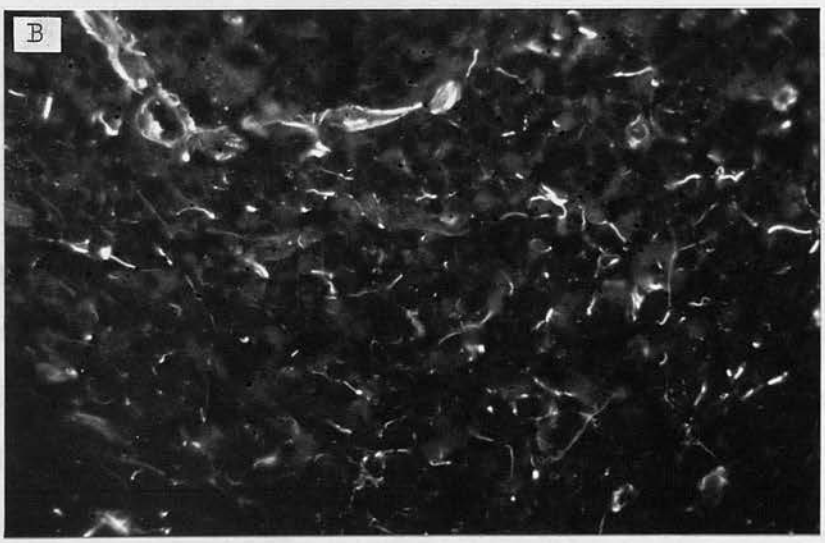
a) Section incubated with Steiner G17 7/15/74 antibody (0.4mg protein/ml) for 30 minutes at room temperature.

b) Section incubated with Steiner G17 7/15/74 antibody (0.2mg protein/ml) for 15 hours at room temperature.

Photographs show granule layer of cerebellum.

Calibration bar = 50/µm





localization of positive staining structures, relative to background i.e. non-specific immunofluorescence. Increasing staining time above 15 hours was not adopted, as it was occasionally found to cause loss of tissue sections from the 'coated' glass slides.

Increased staining times allow localization of histochemical changes in cyclic nucleotides (310), demonstrating that 'stimulated' nucleotides are still detected under these conditions. No advantage was found in increasing the incubation time or dilution of the second antibody (fluorescent conjugate), however.

The working dilution, or immunofluorescent 'titre', must be established for each antibody in each tissue, but may be interpreted differently amongst different workers. For example, one may aim for maximal specific fluorescence by using a low working dilution, and be prepared to accept a degree of 'background' fluorescence. Others may aim, however, for a lower intensity of specific fluorescence with complete absence of 'background', by using a high working dilution of antibody. In the studies reported here however, dilutions of immune and non-immune immunoglobulin have been assessed in parallel on tissue sections, to obtain maximal contrast of specific fluorescence relative to background.

2.3. Fixation

Cyclic nucleotide immunohistochemistry is usually performed on unfixed frozen tissue sections, presumably since the pool of nucleotide that is localized is tightly bound in the tissue section (215). In many immunohistochemical techniques, however, fixation is essential to ensure localization, often by aldehyde perfusion for localization of antigens in the C.N.S. (see reference 311, for example). The effect of various chemical fixatives on cyclic GMP immunohistochemistry is

discussed in more detail in chapter VI, but the results showed that unfixed frozen sections were most suitable, also being quick and simple for tissue processing. Tissue freeze-drying coupled with wax embedding, which increases tissue preservation and prevents any possible diffusion of antigen in the tissue water whilst preparing frozen sections (275), has not been attempted, since it has been shown to reduce the amount of glial fibrillary acidic protein antigen available to antibody, in comparison with that available in frozen tissue sections; this resulted in decreased immunohistochemical sensitivity (312).

3. CYCLIC GMP IMMUNOHISTOCHEMISTRY - CHARACTERIZATION OF POSITIVE STAINING IN THE C.N.S.

3.1. Adult rat

Antibodies generated against succinyl cyclic GMP-HSA or -KLH conjugates showing positive staining, were classified on page 165 as staining fibres, capillaries or both components, in rat cerebellar sections. The following experiments were carried out to characterize these positive staining structures.

3.1.A. Fibre staining

Fibres were stained in all layers of the rat cerebellum, but were particularly marked in the molecular layer where long parallel fibres were observed, frequently running in groups (figure 32), sometimes with a tortuous course (figure 33), and terminating at the external glia limitans at the centre of each folium. This distribution is observed with processes from the Bergmann glial cells whose cell bodies (which were unstained), surround the Purkinje cells (313). In cross-section, the fibres have the appearance of dots (see figure 34, for example). Membranes were observed in many areas surrounding small blood vessels,

a distribution characteristic of glial elements (313).

Characterization of the stained processes as originating from astrocytes was achieved by comparative immunofluorescent studies with an antiserum to the glial fibrillary acidic protein (GFAP), generated in rabbits by Dr A Bignami (314). GFAP is a non-species specific nervous system protein, which has been extensively used as a selective marker for astrocytes in the C.N.S.(313, 314, 315), essentially for the fibrous type, which have long slender processes and differ from protoplasmic astrocytes, which have multiply ramified short processes (316).

Sections of unfixed frozen tissue were processed for immunofluorescence with either Steiner G17 7/15/74 antibody, or GFAP and non-immune sera at a dilution of 1:120, using overnight incubation periods. Identical areas of tissue were then compared and photographed. The results (figures 32, 33, 34, 49) clearly demonstrate the similarity of specific staining, suggesting that the cyclic GMP-positive fibres originate from astrocytes, rather than neurones or other types of neuroglia.

The localization of cyclic GMP-positive fibres and GFAP may be contrasted with that of the glial marker protein S-100 which, for example, is found in the perikaryon of the Bergmann glial cell, in addition to the processes (313).

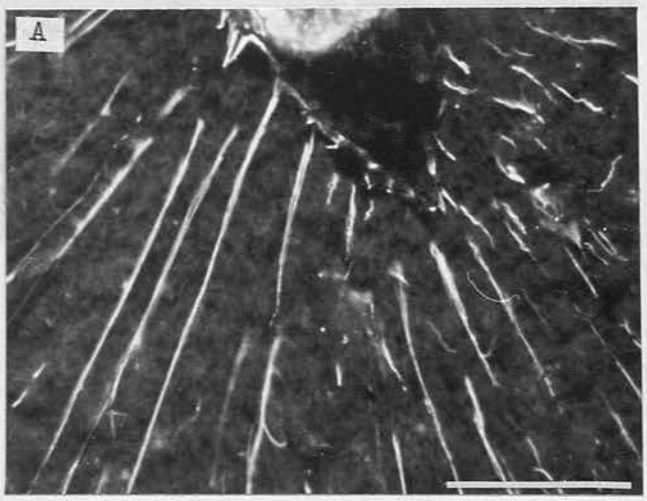
Sections processed from other regions of the C.N.S. (brain stem, substantia nigra and striatum, figures 27, 35 and 36) showed similar localization of cyclic GMP and GFAP positive fibres, often as glial membranes surrounding small blood vessels (see figure 36). A more widespread distribution of staining was observed in white matter areas

Figure 32 Comparative immunofluorescent localization of cyclic GMP and GFAP positive fibres in the molecular layer of the rat cerebellum.

a) Steiner G17 7/15/74 cyclic GMP antibody
b) GFAP antiserum
} Serial sections

c) Steiner G17 7/15/74 cyclic GMP antibody
d) GFAP antiserum
} Serial sections

Calibration bar = 50 µm



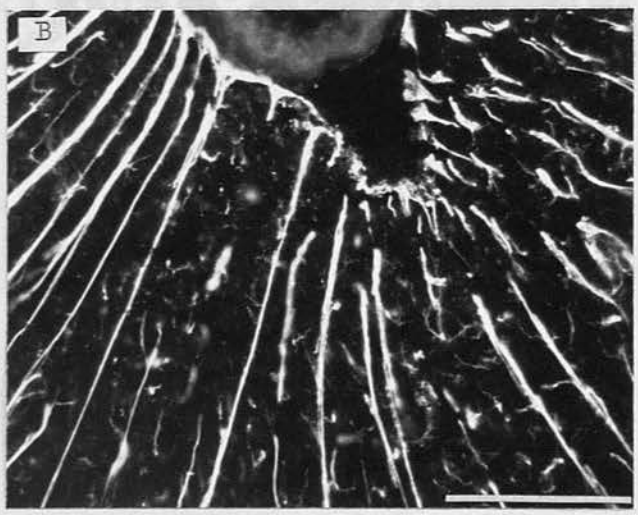


Figure 32 (Continued)

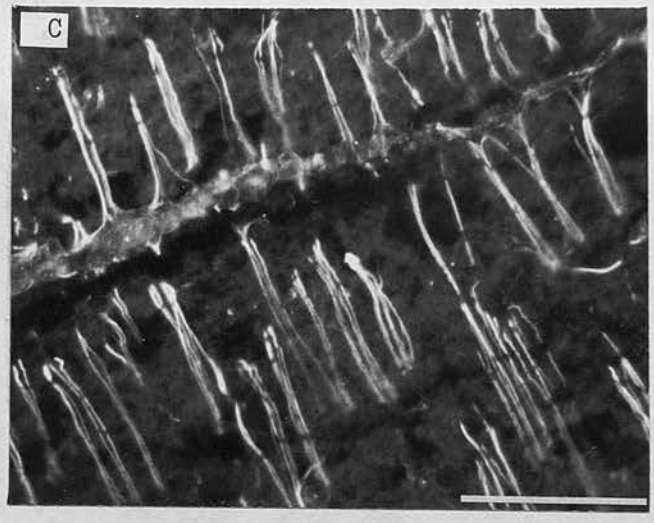


Figure 33 Immunofluorescent localization of cyclic GMP - positive fibres in the molecular layer of the rat cerebellum to demonstrate the tortuous course of the Bergmann glial fibres.

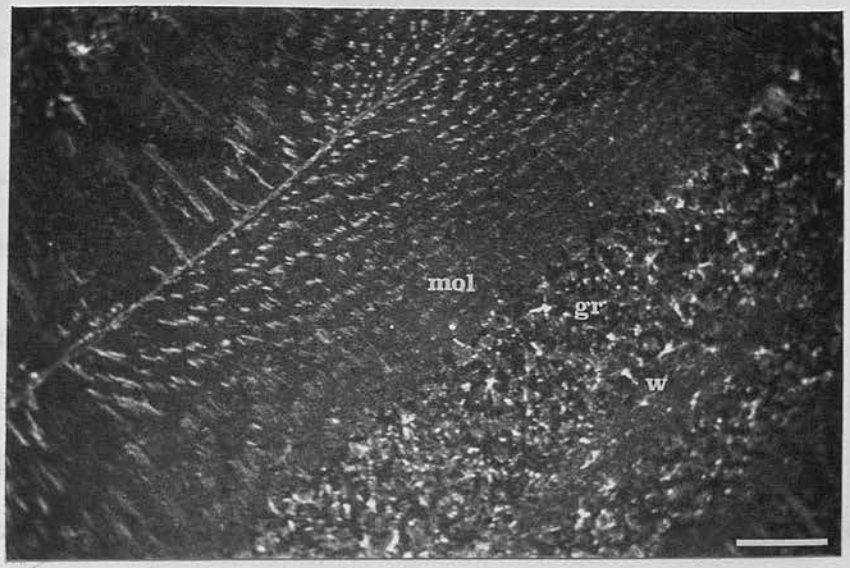
Steiner G17 7/15/74 antibody used for immunofluorescent localization

Calibration bar = 50/µm

Figure 34 Immunofluorescent localization of GFAP in rat cerebellum.

Low magnification. Calibration bar = 100/µm.

gr - granule layer
mol - molecular layer
w - white matter



in the brain as would be expected, since fibrous astrocytes are predominantly found in the white rather than the grey matter (316).

Figure 35 clearly shows the processes of a fibrous astrocyte stained with Steiner G17 7/15/74 cyclic GMP antibody, wrapping around a capillary as the specialized 'end-foot' (316), which attaches to the basement membrane.

Although identical areas were photographed from several sections processed with both antibodies, it is clearly impossible to state categorically that every cyclic GMP positive fibre in a highly complex network such as the cerebellar granule layer or white matter, derives from a fibrous astrocyte. In this situation a 'double-labelling' technique (194) would be beneficial to stain for cyclic GMP and GFAP on the same section with fluorescent anti-species conjugates of different colour; antisera from different species were not available to attempt this, however.

With regard to the distinction of fibrous and protoplasmic astrocytes (313), staining in areas classically considered to contain the protoplasmic type e.g. grey matter of the striatum, has been observed in glial membranes surrounding blood vessels.

Stab-wound injury to the frontal cerebral cortex of rats has been shown to 'switch-on' protoplasmic astrocytes to production of GFAP, and show strong GFAP-positive immunofluorescent staining in these previously unstained glia (317). Stab-wound injury is therefore suggested as a future experiment to determine whether cyclic GMP is involved in this induction of GFAP synthesis in protoplasmic astrocytes.

Figure 35 Immunofluorescent localization of cyclic GMP in the brain stem of the rat.

Steiner G17 7/15/74 antibody used for immunofluorescent localization

Calibration bar = 25 μm

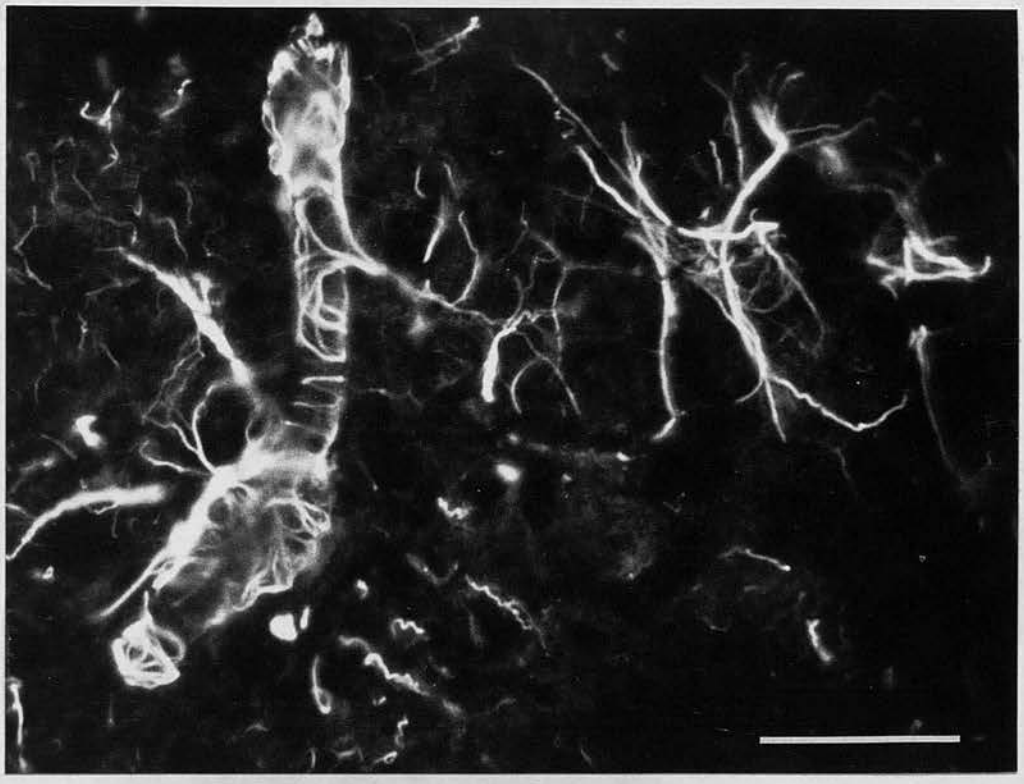
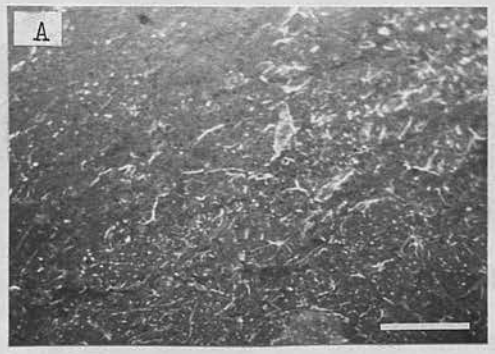


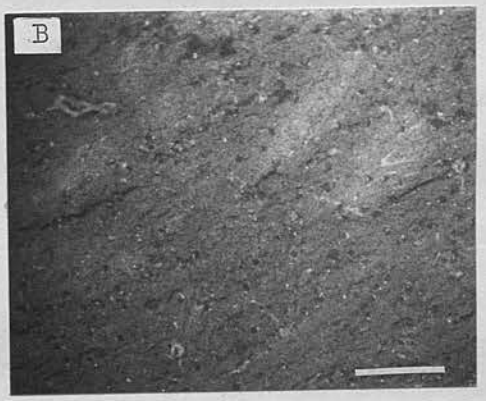
Figure 36 Immunofluorescent localization of cyclic GMP in the substantia nigra and corpus striatum of the rat.

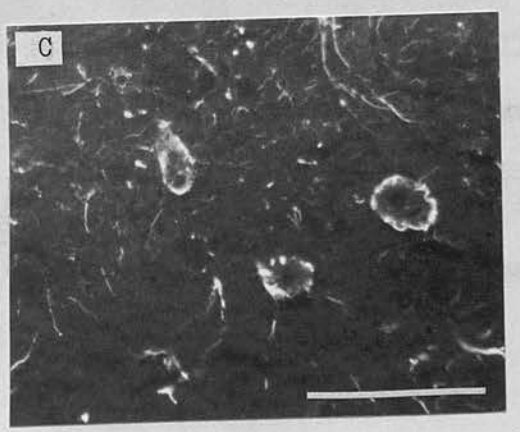
a) Steiner G17 7/15/74 antibody used for localization of cyclic GMP in tissue section containing substantia nigra. Low magnification. Calibration bar = 100 µm

b) Non-immune control for cyclic GMP antibody employed in a). Low magnification. Calibration bar = 100 µm

c) Steiner G17 7/15/74 antibody used for localization of cyclic GMP in the corpus striatum. High magnification. Calibration bar = 50 µm







Future experiments might also examine the ultra-structural localization of cyclic GMP in astrocytes using PAP immunohistochemistry, which may be of higher sensitivity (see page 61), to determine the relation of positive staining structures, to the intermediate size GFAP-positive glial filaments, as visualized by electron microscopy (318).

Having made the distinction between fibre and capillary staining antibodies in rat cerebellum, it was also noted that in one structure in the rat C.N.S., namely the choroid plexus, cyclic GMP staining with 'fibre-staining' antibodies was found with a cytoplasmic distribution in the choroid epithelium (see figure 37). This is a specialized form of epithelium which resembles that of the ependymal (non-nervous) cells which border on the ventricles (316). Comparison with figure 38 showed that these ependymal cells did not stain for cyclic GMP. Note, however, that the sub-ependymal glial network (313) formed from basal processes of the ependymal cells, showed positive cyclic GMP-fibre staining. Non-specific immunofluorescence was particularly noticeable as bright 'dots' at the apex of the ependymal cells, as described in reference 319.

3.1.B. Capillary staining

Capillary wall staining was observed with Steiner G17 12/2/74 and A10 B5 cyclic GMP antibodies in cerebellar tissue sections. Occasionally, bright 'dots' were observed in the capillary walls, probably representing the nuclei of endothelial cells (316) - see figure 39.

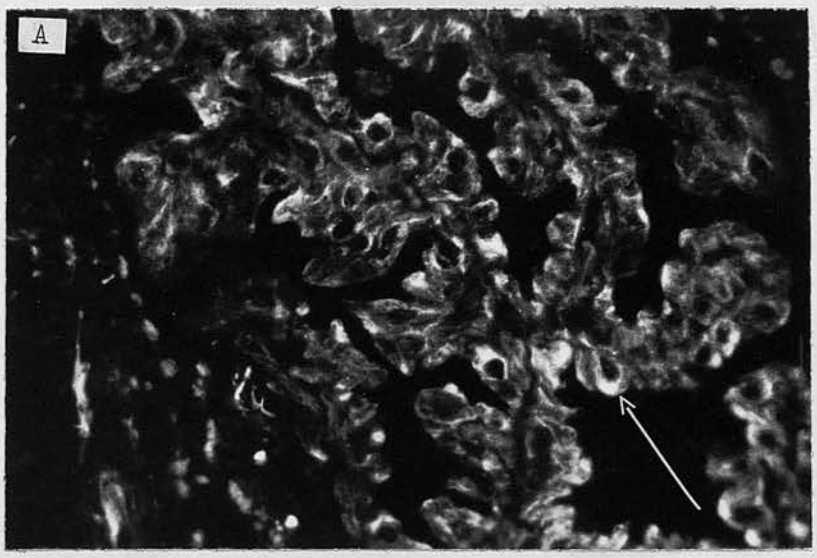
Capillary staining was uniformly distributed in all layers of the cerebellum, and was also found in all brain areas studied, from all species examined in this thesis.

Figure 37 Immunofluorescent localization of cyclic GMP in the choroid plexus.

a) Steiner G17 7/15/74 cyclic GMP antibody. Arrow demonstrates positive cytoplasmic staining of an epithelial cell.

b) Non-immune control for cyclic GMP antibody employed in a). Note non-specific staining at apex of epithelial cells.

Calibration bar = 50μm



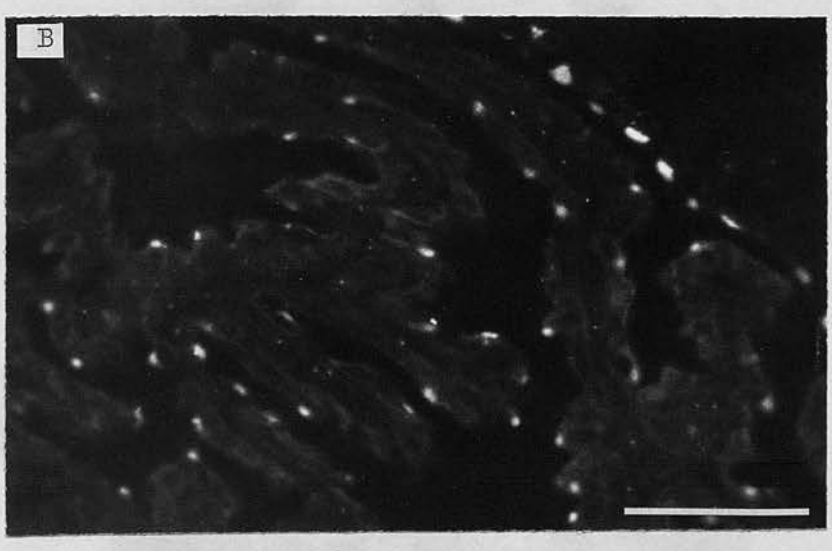
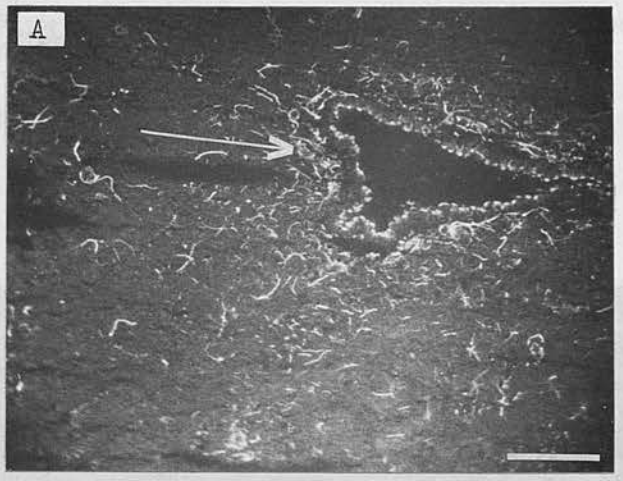
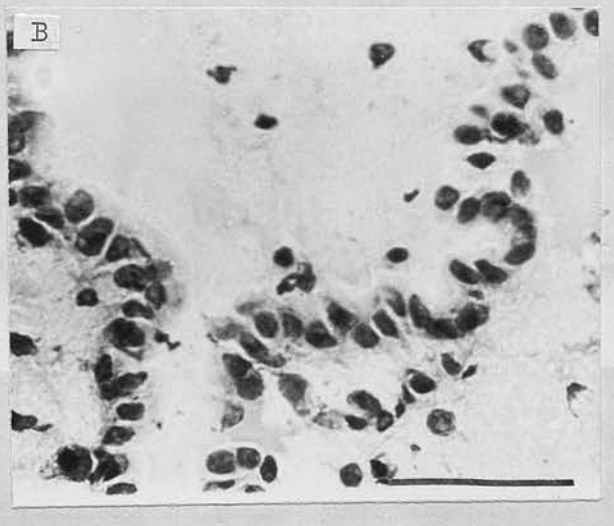


Figure 38 Immunofluorescent localization of cyclic GMP to the sub-ependymal glial fibre network.

a) Steiner G17 7/15/74 cyclic GMP antibody. Low magn. Calibration bar=100 μm
b) Toluidine blue - ependymal cells. High magn. Calibration bar= 50 μm
c) Steiner G17 7/15/74 cyclic GMP antibody. High magn. Calibration bar= 50 μm





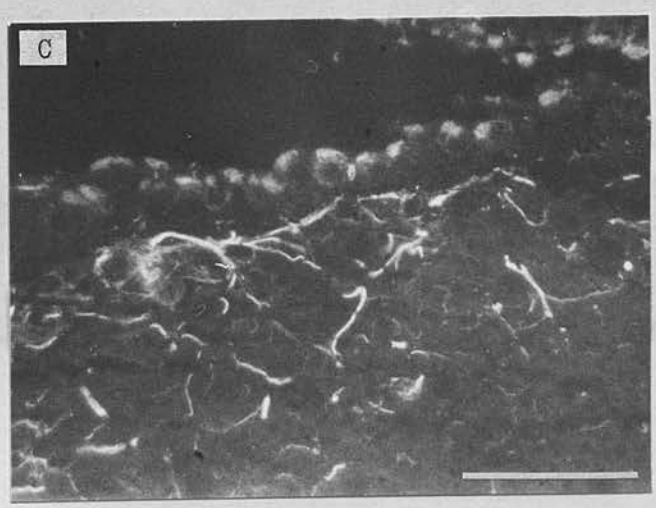


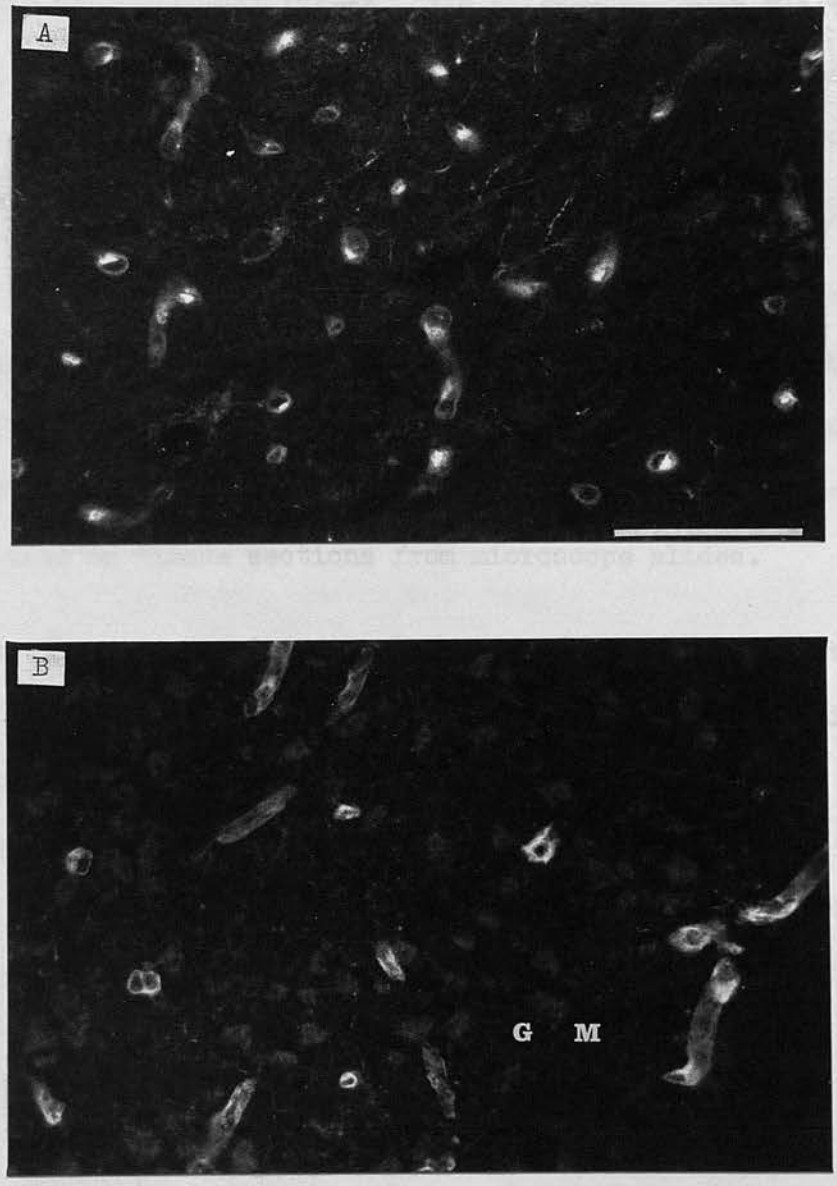
Figure 39 Immunofluorescent localization of cyclic GMP in capillaries to show staining of endothelial cell nuclei.

a) Steiner G17 12/2/74 antibody. Capillaries in cerebellar white matter of the rat.

b) A10 B5 antibody. Capillaries in granule and molecular layers of the rat cerebellum.

Calibration bar = 50μm

G - granule layer
M - molecular layer



3.2. 10 day old rat.

It was considered of particular interest to study the localization of cyclic GMP during development, since cerebellar levels of the nucleotide are 25-30 fold lower in young rats (less than 12 days old) compared with the adult (180). Developing rat cerebellum is also a good model for studying astrocytic proliferation and therefore comparing distribution of cyclic GMP positive fibres with the GFAP antigen.

Six, 10 day old male rats (birth = day 1), from the Bush Estate Animal Breeding Station, Penicuik, Scotland, were employed in these studies. At this stage of post-natal development, rats are without body hair and their eyes are still closed.

Animals were killed by cervical dislocation. Frozen 6μm sections of cerebellum from the young rats, required fixation in 2% freshly depolymerized paraformaldehyde (see appendix 3), followed by buffer washes prior to application of antibody for immunofluorescence processing, to prevent loss of tissue sections from microscope slides.

The peak of astrocytic proliferation occurs during the first post-natal week, after which time the granule layer, as found in the adult cerebellum, forms from migration of the abundant granule cells from the external to the internal granule layer (320, 321). Figure 40 shows the arrangement of cell layers in the cerebellum of a 10 day old rat using toluidine blue histology; this may be contrasted with that of the adult rat cerebellum, as shown in figure 9.

Comparative immunofluorescent localization of cyclic GMP and GFAP positive fibres, showed similar staining patterns in the cerebellum, although important differences were observed. GFAP staining was bright

Figure 40 Comparative immunofluorescent localization of cyclic GMP and GFAP in the cerebellum of a 10 day old rat.

a) Toluidine blue staining to show arrangement of cell layers in 10 day old rat cerebellar cortex. Low magnification. Calibration bar = 100/µm

b) GFAP antiserum. Low magnification. Calibration bar = 100/µm

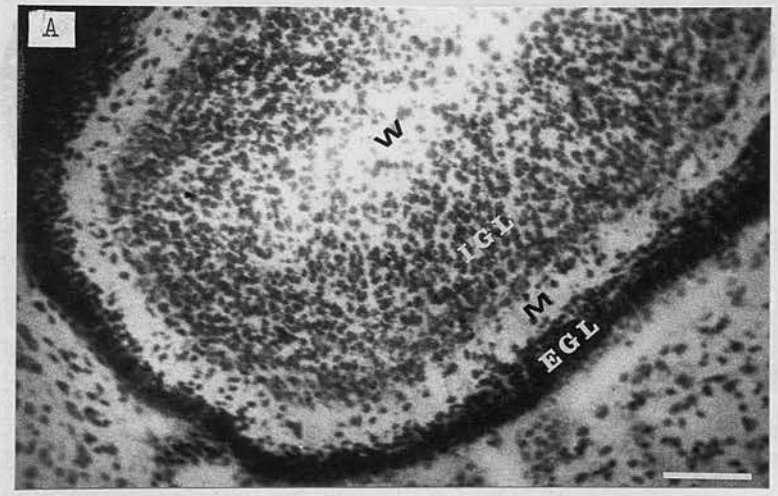
c) Steiner G17 7/15/74 cyclic GMP antibody. Low magnification. Calibration bar = 100/µm

d) GFAP antiserum. High magnification. Calibration bar = 50/µm

Note intense staining of Bergmann glial fibres in the molecular layer.

e) Steiner G17 7/15/74 cyclic GMP antibody. High magnification. Calibration bar = 50/µm

EGL - external granule layer
IGL - internal granule layer
M - molecular layer
P - Purkinje cell body
W - white matter



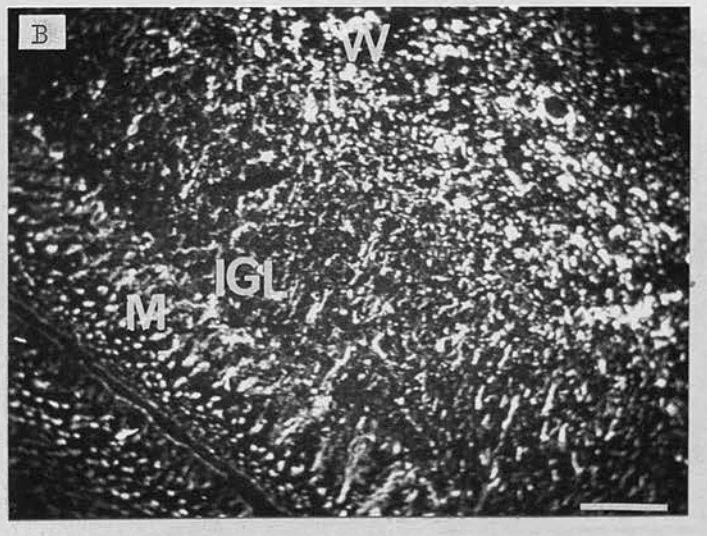
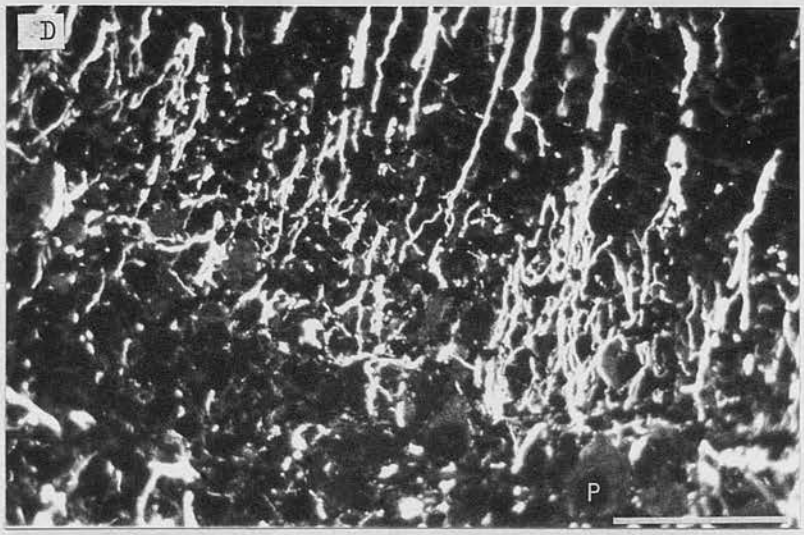


Figure 40 (Continued)



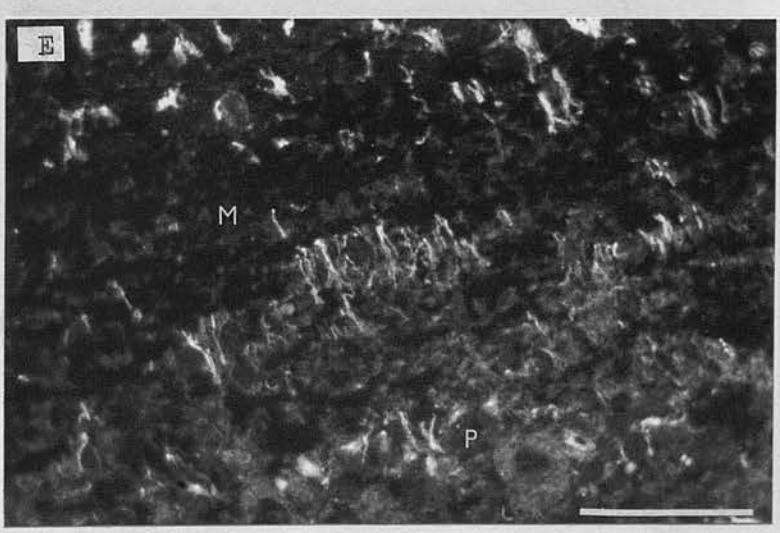
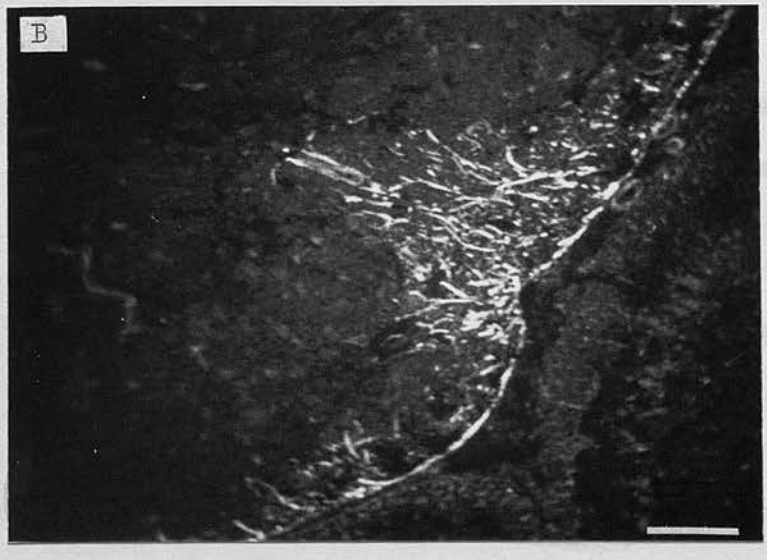


Figure 41 Comparative immunofluorescent localization of cyclic GMP and GFAP in the brain stem of a 10 day old rat.

a) GFAP antiserum

b) Steiner G17 7/15/74 cyclic GMP antibody } Serial sections

Calibration bar = 100 μm



in all layers, whilst cyclic GMP staining was most pronounced in the white matter (see figure 40). The Bergmann glial fibres, for example, showed strong positive staining for GFAP, but were only weakly stained with cyclic GMP antibody. In the brain stem underlying the cerebellum, similar staining was once again observed, although GFAP was more intense and slightly more widespread (see figure 41).

3.3. Adult mouse and rabbit

In view of the variability in response of rat cerebellar slices 'in vitro', as described in the following chapter, biochemical studies were initiated on cerebellar slices from another laboratory animal, namely the mouse. During the course of parallel biochemical and immunohistochemical studies, it was found that cyclic GMP-positive fibres could not be observed in mouse cerebellum, with antibodies showing clear positive fibre staining in rat cerebellum (see figure 52). Antibodies showing capillary localization in the rat however, showed similar localization of these structures in the mouse.

To determine whether astrocytic fibres were present in mouse cerebellum sections were stained with GFAP antiserum, which confirmed the presence of these fibres (see figure 42).

In view of the differential localization of positive staining structures in the rat and mouse cerebellum, sections from two rabbit cerebella (as prepared on page 291) were also processed for immunofluorescence using Steiner G17 7/15/74 cyclic GMP antibody, which showed fibre staining in the rat, but not mouse cerebellum. In the rabbit, fibre staining was observed with a similar localization to that found in the rat; figure 43 contrasts the localization of cyclic GMP and cyclic AMP in rabbit cerebellum. Table 11 summarizes the immunofluorescent localization in rat, mouse and rabbit CNS with different cyclic GMP antibodies

Figure 42 Immunofluorescent localization of GFAP in mouse cerebellar white matter.

Section incubated with GFAP antiserum.

Calibration bar = 50 µm

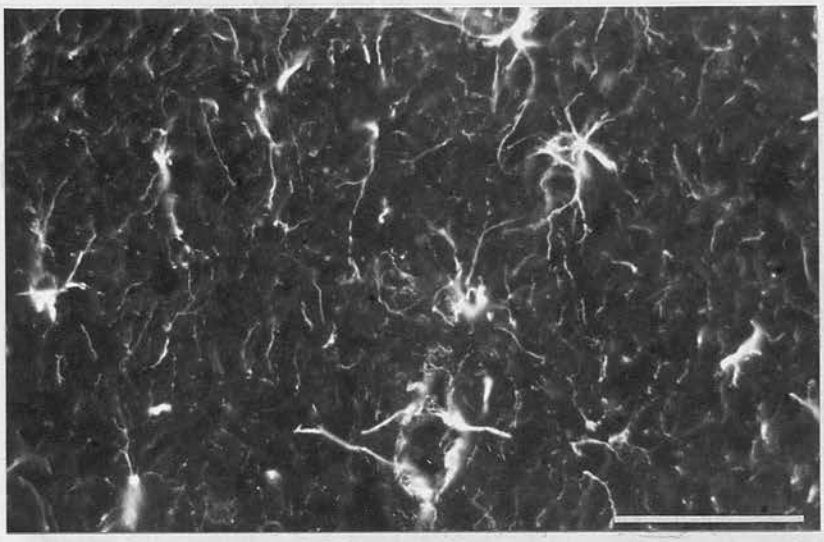


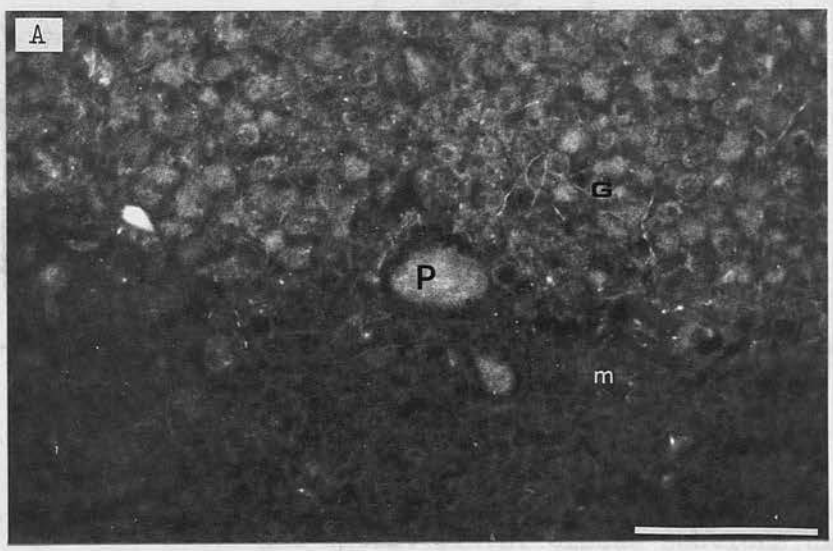
Figure 43 Comparative immunofluorescent localization of cyclic AMP and cyclic GMP in rabbit cerebellum.

a) TEX cyclic AMP antibody. (White dot on photograph represents artefact).

b) Steiner G17 7/15/74 cyclic GMP antibody. Granule layer.

Calibration bar = 50μm

G - granule layer
m - molecular layer
P - Purkinje cell body



ANTIBODY	IMMUNOGEN	SPECIES	FIBRE STAINING	CAPILLARY STAINING
STEINER G17 12/2/74 BLEED	Sc.cGMP-KLH	RAT	✓	✓
		MOUSE	–	✓
		RABBIT	✓	✓
STEINER G17 7/15/74 BLEED	Sc.cGMP-KLH	RAT	✓	–
		MOUSE	–	–
		RABBIT	✓	–
A10 BLEED 5	Sc.cGMP-HSA	RAT	–	✓
		MOUSE	–	✓
		RABBIT	–	✓
A7 BLEED 2	Sc.cGMP-HSA	RAT	✓	–
		MOUSE	–	–

Table 11 Summary of immunofluorescent localization of cyclic GMP in rat, mouse and rabbit C.N.S. tissue sections, using four different cyclic GMP antibodies.

4. CYCLIC GMP IMMUNOHISTOCHEMISTRY - COMPARATIVE STUDIES WITH CYCLIC NUCLEOTIDE ANTIBODIES IN THE LIVER.

It was considered important to determine whether the differences in immunofluorescent localization of cyclic GMP by different antibodies, might be confined to the C.N.S., or observed as a general phenomenon in other tissues. To examine this, immunofluorescence was performed on 6μm frozen sections of rat liver using overnight incubation periods of the cyclic GMP antibodies, at the same dilutions as those employed for localization in cerebellum.

A small piece of liver was removed from a rat and frozen in isopentane - liquid nitrogen approximately 2½ minutes after decapitation. Using identical processing for immunofluorescence, similar staining patterns were observed with Steiner G17 antibodies (bleeds 7/15/74 and 12/2/74) and A7 B2; see figure 44. Whilst hepatocyte membrane staining was observed, as previously reported in the literature for localization of cyclic GMP (215, 305), the intensity of staining was not particularly high, the clearest localization being observed with A7 B2 antibody. This was probably due to the fact that liver tissue required a lower 'working dilution' of antibody for optimal localization of the nucleotide, compared with C.N.S. tissue sections.

Positive staining with cyclic GMP antibodies was not observed in nuclei, althoughthis was previously described by Steiner's group with certain antibodies; weak nuclear membrane fluorescence could be observed with A7 B2 antibody,however. The staining pattern with antibody A10 B5 showed a different localization from the other cyclic GMP antibodies, staining being confined to the endothelial lining of the sinusoids.

Immunofluorescence was also performed with GFAP antiserum,which essentially demonstrated no positive staining, although occasional fibres were visible. This observation suggested that the association of cyclic GMP and GFAP is confined to the C.N.S. and that the nucleotide is associated with fibrous astrocytes, rather than specifically with the marker protein.

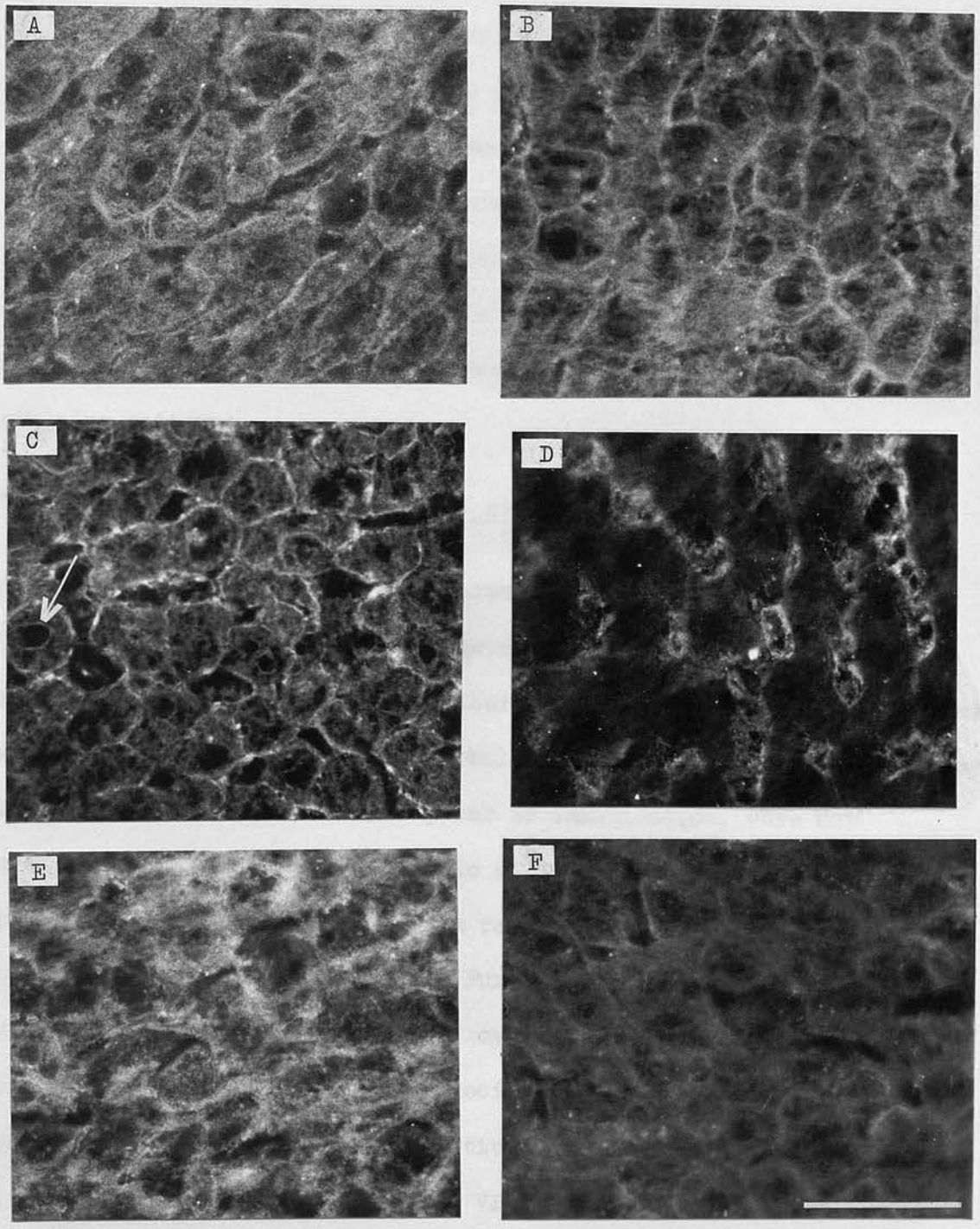
Positive staining with cyclic AMP antibody#7 along the sinusoids, confirmed that demonstrated in the literature (215, 305).

These results in liver, demonstrate that differences in localization with cyclic GMP antibodies,may not be confined to the C.N.S.

Figure 44 Immunofluorescent localization with cyclic nucleotide antibodies in rat liver.

a) Steiner G17 7/15/74 cyclic GMP antibody.
b) Steiner G17 12/2/74 cyclic GMP antibody.
c) A7 B2 cyclic GMP antibody - nuclear membrane arrowed.
d) A10 B5 cyclic GMP antibody.
e) #7 cyclic AMP antibody.
f) Non-immune immunoglobulin.

Calibration bar = 50 μm



5. CYCLIC GMP IMMUNOHISTOCHEMISTRY - SPECIFICITY.

Specific immunofluorescent staining is primarily dependent upon the defined immunological reaction between the first antibody and the tissue antigen, in the indirect technique (194) . Whilst non-specific fluorescence due to non-immunological reactions has already been considered in detail, it is necessary to demonstrate that removal of either specific antibody, or specific antigen from the tissue section, results in elimination of staining (302, 303). The following discussion considers methods used for testing this immunological specificity. Stringent tests are required for cyclic nucleotide antibodies, since only a small number show positive immunofluorescence, and even these may show different staining patterns.

5.1. To demonstrate that positive staining was due to antibodies generated against the immunizing conjugate.

Staining patterns in cerebellar tissue sections were examined with immunoglobulin fractions of pre-immune sera and sera taken during the course of immunization, from a number of rabbits. The lack of positive staining with pre-immune immunoglobulins, demonstrated that circulating rabbit anti-tissue antibodies prior to immunization, were not responsible for staining. Specific staining structures were only localized with certain bleeds from rabbits, obtained after repeated booster injections of immunogen. Furthermore,immunization of a large number of rabbits with cyclic AMP, cyclic GMP kinase or catalytic sub-unit immunogens in the same species of rabbit, by an identical procedure, failed to show the positive staining structures found with cyclic GMP antibodies (see chapter VI).

5.2. To demonstrate that positive immunofluorescent staining was not a result of antibodies raised against the carrier protein employed for immunization.

In cyclic nucleotide RIA, only the population of antibody molecules that binds tracer is under investigation, whereas for cyclic nucleotide immunohistochemistry, the population of antibodies directed towards the carrier protein could also bind this protein, or cross-reacting antigens, in the tissue section.

To demonstrate that immunization of rabbits with hapten-protein conjugates results in antibodies raised against not only the hapten, but also the protein, antibody A7 B2 which had been shown by RIA to have high specificity antibodies against cyclic GMP, was tested for antibodies against the carrier molecule, human serum albumin (HSA), and the related protein, bovine serum albumin (BSA). Agarose immunodiffusion plates were obtained from Miles Laboratories, Slough, Buckinghamshire (code no. 64-275). 20µl of undiluted antibody (immunoglobulin fraction) were placed in the centre well, and varying concentrations of the two proteins (in PBS) were placed in the six outer wells, in the same sample volume. After 72 hours at room temperature, the plates were viewed. With a concentration of HSA as low as 0.06mg/ml, a white line was observed between the centre hole and peripheral hole, demonstrating the presence of antibody. Using BSA, however, no line was observed with protein concentration as high as 10mg/ml, the highest concentration tested in this experiment. These results demonstrate the specificity of the antibodies generated against the carrier protein.

The possibility that antibodies against the carrier protein were responsible for immunofluorescent staining in tissue sections, was considered unlikely, since fibre and capillary staining was observed

with antibodies coupled to either HSA or KLH, demonstrating that the conjugation procedure did not effect staining pattern. However, antibodies A7 B2 and A10 B5 were incubated overnight with 10mg/ml HSA at 4°C, to cause precipitation of the antibody-carrier complex (311). After centrifugation at 13,000g for 3 minutes (Eppendorf centrifuge), the 'absorbed' antibodies were compared with 'unabsorbed' antibodies, diluted in PBS at the same immunoglobulin concentration; no significant effect was observed on specific staining. As a further control, sections were incubated with a range of dilutions of commencial rabbit antisera to HSA (supplied by Nordic Immunologicals, Maidenhead, Berkshire); no staining was observed in tissue sections.

5.3. To demonstrate that positive staining was due to cyclic GMP in the tissue section.

Antibodies raised against succinyl cyclic AMP-HSA showed entirely contrasting patterns to those observed against succinyl cyclic GMP-HSA. This demonstrates that a non-specific antibody has not been raised against a component of the nucleotide-carrier linkage, causing positive immunofluorescent staining. This suggests that the cyclic GMP portion of the immunogen, in particular the purine determinants, are responsible for specific staining and the contrasting localization of cyclic AMP with cyclic GMP.

RIA cross-reactivity data has demonstrated that the cyclic GMP antibodies used for immunofluorescence are specific, and do not show any appreciable cross-reactivity to related nucleotides. The following sections, however, consider methods of removing cyclic GMP antibody to satisfy the more precise immunohistochemical criteria of specificity (302, 303).

5.3.A. 'Liquid-phase'blocking test.

This test is often used to demonstrate specificity in immunohistochemistry (194). Antigen is added to antibody in solution, and in cases where the antigen-antibody complex precipitates e.g. protein antigens, complex may be removed by centrifugation, and the resulting supernatants,free from specific antibody, used to demonstrate removal of specific immunofluorescence. This technique has been used for cyclic nucleotide immunohistochemistry with precipitating antibodies that are raised in goats (310). In cases where immune complexes are soluble e.g. rabbit cyclic nucleotide antibodies (215), specific antibody is not removed, but remains blocked by excess antigen, and application of this blocked antibody to the tissue section should prevent specific staining.

Steiner et al (215) have carried out'liquid-phase' blocking experiments in tissues such as liver,with cyclic nucleotide antibodies, and reported that low concentrations ($1/\mu$M) of the specific cyclic nucleotide could prevent staining, whereas higher concentrations of related nucleotides failed to prevent staining.

Similar experiments have been performed in the laboratory using A7 B2, A10 B5 and Steiner G17 antibodies, incubated with concentrations of cyclic GMP as high as 1mM (of the sodium salt and the free acid), without producing any effects on specific staining in cerebellum or liver, however, using 30 minute or overnight incubation periods.

To confirm that cyclic GMP antibody was effectively blocked after 15 hour incubation at 4^{o}C with excess antigen, the binding capacity of antibodies were measured, as described on page 141 • The results in table 12 demonstrate that as little as $1/\mu$M cyclic GMP used for

pre-incubation caused almost complete loss of binding for [^{3}H] cyclic GMP, indicating that available cyclic GMP sites were blocked by this procedure.

Table 12 Effect of pre-incubation of cyclic GMP antibodies (15 hours, 4°C.) with concentrations of unlabelled cyclic GMP and cyclic AMP, on subsequent binding of antibodies to [^{3}H] cyclic GMP in assay.

CYCLIC GMP ANTIBODY (IMMUNOGLOBULIN FRACTION)	ANTIBODY PRE-INCUBATED WITH cGMP/cAMP	PERCENTAGE [^{3}H] cGMP BOUND
A10 B5	-	40.5
	10^{-3}M cGMP	0.6
	10^{-4}M cGMP	0.6
	10^{-6}M cGMP	1.2
	10^{-6}M cAMP	39.9
A7 B2	-	32.3
	10^{-3}M cGMP	0
	10^{-4}M cGMP	0.7
	10^{-6}M cGMP	1.0
	10^{-6}M cAMP	25.8
STEINER G17 7/15/74	-	22.7
	10^{-3}M cGMP	0.6
	10^{-4}M cGMP	0
	10^{-6}M cGMP	0.7

Percentage binding values shown in table, calculated after subtraction of non-specific binding(with non-immune immunoglobulin)

Antibodies were also incubated in'liquid-phase'with 1 mM succinyl cyclic GMP and 1mg/ml of immunizing conjugate. Despite this, no reduction was found in the distribution or intensity of specific staining.

Incubation of Steiner G17 12/2/74 with 15 mM cyclic GMP under the same conditions, resulted in removal of capillary staining, whilst having no effect on fibre staining (see figure 45). Although this is a high concentration of cyclic GMP, and the specificity of this block must therefore be of question, incubation with 15 mM cyclic AMP had no effect on either capillary, or fibre staining, demonstrating a degree of immunological specificity for the cyclic GMP block. To determine whether the capillary component of A10 B5 antibody showed blocking in a similar fashion with 15 mM cyclic GMP under these conditions, the experiment was repeated with this antibody; no effect was observed on capillary immunofluorescence, however.

To ensure that staining, assumed to be due to cyclic GMP, could not be removed by incubating antibodies in 'liquid-phase' with related nucleotides, similar experiments were carried out by incubating Steiner G17, A7 B2 and A10 B5 antibodies, with cyclic AMP, cyclic IMP, cyclic UMP and 5' GMP; no effect was found on immunofluorescent staining.

The effect of increasing incubation time of antibodies with cyclic GMP was also observed, without effect, together with one hour incubation at 37°C prior to overnight incubation. Similar results using 'liquid-phase' blocking to those previously described, were observed with #7 and TEX cyclic AMP antibodies.

The possibility was considered that staining was not being removed, since immune-complexes being applied to tissue sections in blocked antibodies, might be able to bind unoccupied cyclic GMP 'receptors' in the tissue, and therefore show positive staining; this effect has been observed with LH-RH immunohistochemistry (218). To examine this possibility, sections were incubated for 45 minutes at room temperature

Figure 45 Effect of pre-incubation of Steiner G17 12/2/74 cyclic GMP antibody with 15mM cyclic AMP or 15mM cyclic GMP, prior to immunofluorescent staining in rat cerebellum.

a)) Pre-incubation with 15mM cyclic AMP
b)) Pre-incubation with 15mM cyclic GMP
)) Calibration bar = 50 µm

P - Purkinje cell body

with 1 mM or 10 mM cyclic GMP to saturate any unoccupied sites, followed by washing in buffer and immunofluorescence processing. No difference was found between sections pre-incubated with cyclic GMP or buffer (see figure 59). Similar results were also observed with TEX cyclic AMP antibody after pre-incution of sections with cyclic AMP.

'Liquid-phase' blocking experiments, whilst widely used, have also been found by a number of workers to be unsuitable specificity controls for non-precipitating antibodies. For example, Swaab and Pool (300), have found pre-incubation ineffective and suggest that antibodies which are bound reversibly to free antigen during pre-incubation, 'prefer' the tissue-bound antigen, to the antigen in the medium.

In studying cyclic nucleotide antibodies, Steiner et al (215) have reported that whilst some antibodies will block in 'liquid-phase', others will not, although all antibodies show the same staining patterns. This has also been observed by Rosenberg et al (personal communication).

5.3.B. 'Solid-phase' blocking test.

i) Introduction.

An alternative approach to 'liquid-phase' blocking, is removal of specific antibody by absorption with antigen that is bound to an insoluble matrix (e.g. 215, 300). The technique of affinity chromatography enables antigen to be bound covalently in the form of a gel column; passage of antibody through the column enables specific antibody to bind, and be eluted under appropriate conditions of buffer strength and pH, which cleave the antigen-antibody bond. 'Solid-phase' techniques which are frequently used to prepare monospecific antibodies (280), might be expected to resemble immunohistochemical conditions, since antigen is immobilized in both cases. Purified antibodies, however, may be of low titre and be unstable (322).

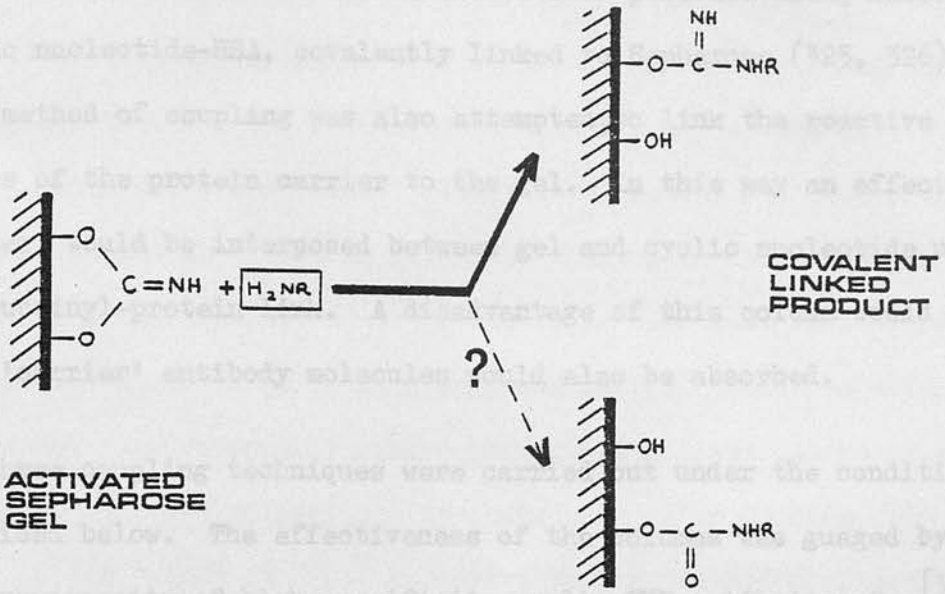


Figure 46 Proposed mechanism for coupling primary amino groups to 'activated' Sepharose. Redrawn from (323).

Cyclic AMP and cyclic GMP possess a free amino group on the purine nucleus (N6 and N2 positions respectively), which is available for coupling to an 'activated' form of Sepharose. Cyanogen bromide activation is most commonly employed, for coupling (see figure 46).

The coupling procedure was attempted, as successfully employed by Fallon et al (324), for linking cyclic nucleotides to Sepharose. For small molecules linked covalently to Sepharose, 'spacer' groups are often substituted between the molecule and the gel. Six carbon 'spacers' are often used, and by increasing the distance of the molecule from the gel, increase the efficiency of antibody binding, by reducing steric hindrance (323). Affinity columns were therefore also constructed of cyclic nucleotides linked to activated Sepharose with a six carbon 'spacer'.

Cyclic nucleotide antibodies have also been purified using succinyl cyclic nucleotide-HSA, covalently linked to Sepharose (325, 326). This method of coupling was also attempted, to link the reactive amino groups of the protein carrier to the gel. In this way an effective 'spacer' would be interposed between gel and cyclic nucleotide using the succinyl-protein link. A disadvantage of this column would be that 'carrier' antibody molecules would also be absorbed.

The three coupling techniques were carried out under the conditions described below. The effectiveness of the columns was guaged by the binding capacity of high specificity cyclic GMP antibodies for [^{3}H] cyclic GMP, eluted from 'control','cyclic AMP' and 'cyclic GMP' affinity columns.

ii) Production of affinity columns.

The following procedure (based on reference 324), describes the linking of cyclic AMP and cyclic GMP to'activated' Sepharose; identical procedures were carried out for production of the other affinity columns.

Cyanogen bromide - 'activated' Sepharose 4B and 'activated' ACH-Sepharose 4B (Sepharose with six carbon 'spacer'), were obtained from Sigma Chemical Company, Norbiton, Surrey. Pre-activated gels were used since cyanogen bromide activation is extremely hazardous.

10g of the freeze-dried gel were suspended in 50ml of 1 mM HCl, and stirred gently with a glass rod to remove any 'clumps' of gel. A glass sinter funnel(grade 3)was washed through several times with the acid. The suspended gel was transferred to the sinter, and washed through with 1 litre of 1 mM HCl, to ensure removal of dextran and lactose preservatives in the gel. 400ml of distilled water were then passed through the gel, followed by 1 litre of 0.1M bicarbonate buffer, pH 9.0.

Three test tubes were each filled with 10ml of gel (packed volume). 1.5ml of a solution of either cyclic AMP, or cyclic GMP (50mg sodium salt, Sigma Chemical Co, dissolved in coupling buffer and adjusted to pH 9.0), or buffer, were added. Tubes were tightly stoppered, and shaken overnight at 4°C on an automatic shaking machine (Luckham Ltd, England). After incubation, the contents of each tube were washed into three glass sinter funnels, with coupling buffer. Prior to use, the sinters had been thoroughly washed through with distilled water, and phosphate-buffered saline (see appendix 2). After washing, gels were stored in the buffer at 4°C. Preservative was not added, since the gels were used, without recycling, within one week of preparation.

In order to test the cyclic nucleotide-Sepharose columns, 1ml of each gel (packed volume) was placed in a disposable column. Columns were constructed from glass Pasteur pipettes containing a small 'plug' of cotton wool at the point where the pipette narrows, and a short length of polypropylene tubing, attached to the end of the narrow glass tube. Flow rates were made equivalent between columns, using small adjustable clips on the polypropylene tubing. Assembled columns were mounted on a rack and washed through with 5ml of PBS. 300µl of rabbit non-immune immunoglobulin (supplied by Mercia Diagnostics, and diluted 1:10 with PBS) were then added to each column, followed by washing through with 4ml of buffer. Non-immune rabbit immunoglobulin was used to block any non-specific reactive sites, which might have been present on the gel as a result of incomplete conjugation. 300µl of diluted cyclic GMP antibody were then layered onto each column, and washed through with buffer. Eluate was collected from each column after addition of specific antibody, together with 4ml of buffer washings to remove any loosely bound antibody.

Samples were dialyzed (as described on page 121) at 4°C for 18 hours vs. distilled water. Dialyzed samples were then freeze-dried (Leybold-Heraeus GT2 freeze-dryer, Germany), and reconstituted with 300µl of PBS. 50µl aliquots of each antibody were then assayed under identical conditions, for their ability to bind [^{3}H]cyclic GMP, as described on page 141. Since some losses occurred during passage through Sepharose columns, binding of antibodies from 'control' columns was compared with those from 'cyclic AMP' and 'cyclic GMP' columns.

iii) Results.

The results shown in table 13 demonstrate that whilst antibody to cyclic GMP is removed by passage through 'cyclic GMP' affinity columns, significant amounts are also removed on passage through 'cyclic AMP' columns, although by RIA these antibodies show neglible cross-reactivity to cyclic AMP. In order to check that the cyclic nucleotide -Sepharose was neither saturated with antibody running through the column, nor only partially accessible to antibody, aliquots of gel were incubated with antibody in Eppendorf tubes. After pre-blocking with non-immune immunoglobulin, gel was incubated with antibody for 30 minutes at room temperature, with frequent mixing. After incubation, gel was allowed to settle, and an aliquot of antibody tested for binding to [^{3}H]cyclic GMP. Similar results were obtained as with the column procedure.

iv) Discussion

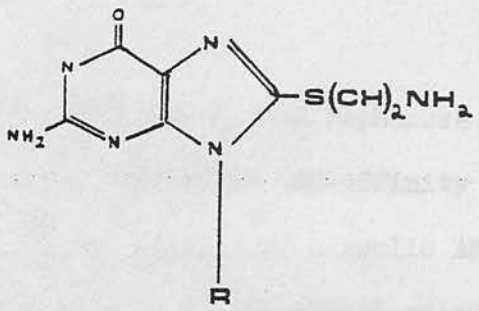
The results demonstrate that the affinity columns are not selectively removing a population of antibodies specifically directed towards cyclic GMP, when the purine amino group of the cyclic nucleotide is coupled to the matrix. Similar results were also obtained with linkage of the immunogen by the free amino groups of human serum albumin; it is assumed that the immunogen links via protein amino groups rather than the free purine amino group of the cyclic nucleotide. The low reactivity of this

Table 13 Binding of cyclic GMP antibodies to [^{3}H] cyclic GMP after passage through cyclic nucleotide affinity columns.

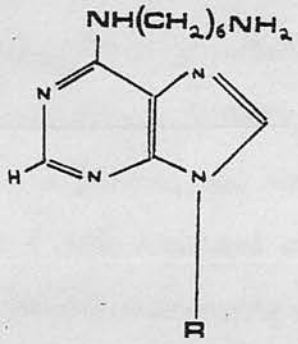
AFFINITY MEDIA	A10 B5 ANTIBODY		A7 B2 ANTIBODY	
	% [^{3}H] cGMP BOUND BY COLUMN ELUATE	% LOSS OF cGMP ANTIBODY RELATIVE TO ELUATE FROM CONTROL	% [^{3}H] cGMP BOUND BY COLUMN ELUATE	% LOSS OF cGMP ANTIBODY RELATIVE TO ELUATE FROM CONTROL
CONTROL - ACTIVATED SEPHAROSE	24.8		17.4	
cGMP - ACTIVATED SEPHAROSE	0	100	0	100
cAMP - ACTIVATED SEPHAROSE	8.3	64.8	2.0	88.5
cGMP-'SPACER' - ACTIVATED SEPHAROSE	0	100		
cAMP-'SPACER' - ACTIVATED SEPHAROSE	5.3	77.5		
SUCCINYL cGMP-HSA - ACTIVATED SEPHAROSE	0	100		
SUCCINYL cAMP-HSA - ACTIVATED SEPHAROSE	6.4	72.9		

Percentage binding values shown in table, calculated after subtraction of non-specific binding with non-immune immunoglobulin in assay.

Figure 47 Two cyclic nucleotide derivatives that have been used for affinity chromatography; see references 328 and 330.



8-(2-aminoethyl)-thio-cGMP



N^6-aminohexyl-amino-cAMP

---

amino group on cyclic AMP, has been commented upon previously (327). Derivatives of cyclic AMP have been synthesized, and used to form a more highly reactive amino group for conjugation to Sepharose; for example, N^6 - aminocaproyl cyclic AMP (327) and N^6 - aminohexyl-amino cyclic AMP (328); see figure 47.

Cyclic AMP has also been linked to Sepharose at the C-8 position (328, 329); linkage at this position makes possible the synthesis of any purine nucleotide - Sepharose conjugate. Recently, cyclic GMP has been immobilized for affinity chromatography of cyclic GMP-dependent protein kinase (330), by synthesis of 8-(2-aminoethyl)-thio-cyclic GMP linked to Sepharose; see figure 47 above.

C8-nucleotide linked affinity columns might prove to be of higher specificity, and should therefore be considered for preparation in future studies.

In these experiments cyclic AMP-linked Sepharose has been used to guage the selectivity of the cyclic GMP-affinity columns. Rosenberg et al (331) have shown that passage of a cyclic AMP antibody through an agarose affinity column of N^6-aminohexyl-amino-cyclic AMP, does not produce a specific cyclic AMP antibody, as judged by cross-reactivity of eluted antibodies to related adenosine nucleotides.

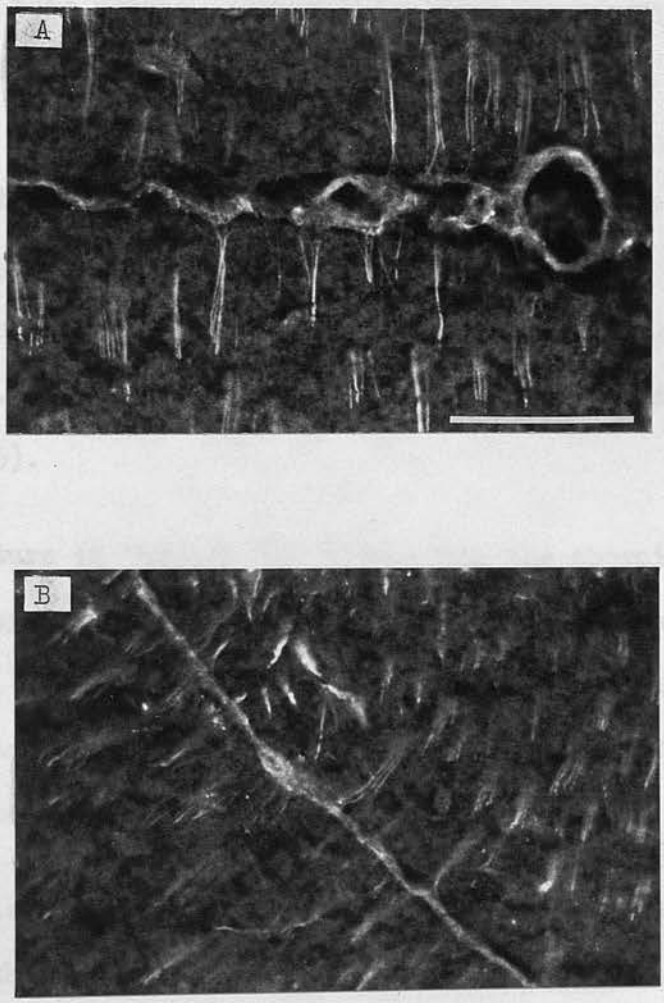
To determine whether matrix-linked cyclic nucleotides prevented immunofluorescent staining by absorbed antibodies however, reconstituted antibody (A7 B2) from affinity columns of cyclic AMP-activated Sepharose, cyclic GMP-activated Sepharose, and control-activated Sepharose, as prepared on page 221 , were examined at equivalent immunoglobulin concentrations, by immunofluorescence on rat cerebellar sections. Figure 48 shows that there was no significant difference in immunofluorescent staining patterns with the column eluates, however.

Figure 48 Immunofluorescent localization of cyclic GMP in the molecular layer of the rat cerebellum using A7 B2 antibody, after passage through control and cyclic GMP affinity columns.

a) Reconstituted antibody eluate from control Sepharose column.

b) Reconstituted antibody eluate from cyclic GMP-Sepharose column.

Calibration bar = 50 µm



6. GENERAL DISCUSSION

Comparative immunofluorescent studies with fibre-staining cyclic GMP antibodies and the astrocytic marker protein, GFAP, revealed similar localization in adult rat C.N.S., and when applied to the cerebellum and underlying brain stem of the 10 day old rat. For similarities of GFAP and myosin immunofluorescence, which will be discussed in more detail in chapter VI, it has been reported (as found in these studies comparing GFAP and cyclic GMP), that both antigens show similar staining, although GFAP is 'sharper' (332). This has been attributed to myosin not being totally astrocyte-specific, and a similar argument may hold for comparison of cyclic GMP and GFAP fibre staining, since cyclic GMP immunofluorescence is not restricted to the C.N.S. (e.g. localization in liver in these studies; see also reference 305).

In one structure in the rat C.N.S. however, the choroid plexus epithelium, cyclic GMP fibre-staining antibodies showed a cytoplasmic localization, which was not found in the more unspecialized ependymal cells bordering the ventricles, perhaps suggesting a functional role in this structure (see chapter VI).

Having postulated that cyclic GMP antibodies contain populations of molecules with different avidities for tissue-bound nucleotide associated with different structures, a further degree of complexity is raised by the results in this chapter demonstrating that the fibre-staining component is observed in rat and rabbit C.N.S., but not in mouse C.N.S. This indicates that the particular configuration of tissue-bound cyclic GMP is either absent, or below the sensitivity of immunofluorescent detection in the mouse; further experiments will be required to determine whether this is a definite species, or perhaps strain effect.

Preliminary studies with a small number of cyclic AMP antibodies from Dr Steiner's laboratory have demonstrated that differences in binding of this nucleotide in the tissue section, may also cause differences in localization, in a similar way to cyclic GMP. The completely contrasting localization of the two nucleotides, however, emphasizes the degree of resolution that may be achieved by the technique, between structurally related nucleotides at different tissue sites.

Proof of specificity of cyclic GMP immunofluorescence observed with a small number of positive staining antibodies, is complicated by the failure of the classical tests of antibody removal to abolish immunofluorescent staining. Certain antibodies, for example, will specifically block staining when incubated with excess cyclic GMP in 'liquid-phase', whereas others will not, although both types will demonstrate identical staining patterns (215). Similarly, certain cyclic GMP antibodies fail to block staining, even when passed through columns containing the nucleotide covalently linked to Sepharose gel.

Applying the concept of stereo-specific differences in tissue-bound cyclic GMP, the population of antibodies useful for immunofluorescence, may have higher avidity for tissue-bound cyclic GMP than for nucleotide in solution, or even immobilized on a solid-phase matrix, under the conditions described in these experiments.

RIA cross-reactivity studies have demonstrated that cyclic GMP antibodies showing positive immunofluorescence, have high specificity for cyclic GMP in solution. Since the immunohistochemical specificity controls are incomplete, however, there exists the possibility that the antigen being localized in tissue sections may not be cyclic GMP. Future production

of rabbit cyclic nucleotide antibodies, must aim to select positive staining antibodies which are specifically blocked by'liquid'or'solid'-phase techniques. Of greater interest would be specific cyclic nucleotide affinity matrices, which could remove staining, since they would indicate the configuration of bound nucleotide in the tissue section. Sub-cellular fractionation studies might also help to confirm immunofluorescent localization, and determine whether cyclic GMP is present in hitherto undetected sites (discussion on the bound pool of cyclic GMP in tissue sections is presented in chapter VI).

A number of other observations suggest that cyclic GMP antibodies are localizing the tissue-bound nucleotide:

i)Buffer-washed tissue sections, in which greater than 80% of cyclic GMP is lost (see chapter V), have a residual pool of cyclic GMP as detected by RIA of extracted tissue.

ii) Initial observations using an enriched astrocytic fraction from 8 day old rat cerebellum (321), have shown significant levels of cyclic GMP (approximately 10p mole^{5}/mg of protein) compared with levels of approximately 2p mole^{5}/mg of protein, in a mixed cell population (personal communication, Dr J Garthwaite). Cyclic GMP has also been detected in vascular endothelial cell cultures from the intima of rabbit aorta (167). Since capillaries are tubes of endothelial cells surrounded by a thin basal lamina (316), this observation may be of significance.

Attempts to alter tissue levels of cyclic GMP, to correlate with altered intensity or distribution of cyclic GMP immunofluorescence, are considered in the following chapter.

CHAPTER V

ATTEMPTS TO CORRELATE BIOCHEMICAL CHANGES IN CYCLIC GMP TISSUE LEVELS IN THE CEREBELLUM, WITH IMMUNOHISTOCHEMICAL STUDIES

CHAPTER V

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1. MEASUREMENT OF TISSUE LEVELS OF CEREBELLAR CYCLIC GMP, USING RIA.

In order to determine whether immunohistochemistry could localize biochemical changes in cyclic GMP to specific sites in the cerebellum, an assay was established for measuring tissue levels, based on the RIA technique previously described in chapter III.

1.1. Preparation of tissue samples for assay

Male, adult Wistar rats of approximately 200g, and male, adult albino CFI mice of approximately 35g, were obtained from the Bush Estate Animal Breeding Station, Penicuik, Scotland.

For measurement of 'in vivo' cyclic GMP levels, approximately 20mg of cerebellar tissue were removed from the animal at a specified time after decapitation, washed briefly in 'ice-cold' physiological saline, and homogenized with 200µl of 80% ethanol/water in 1.3ml plastic Eppendorf tubes using a Teflon pestle of 0.39" diameter (homogenizer, Gallenkamp, Glasgow). For 'in vitro' studies, slices were removed from the incubation medium and immediately homogenized. Following 30 seconds of homogenization, 1ml of 80% ethanol/water was added to the tubes, which were then vortex mixed. It was important to ensure that homogenization was vigorous, but did not cause spillage of samples. Samples were allowed to stand for approximately one hour before being evaporated to dryness overnight on a 'Dri-Block', at 60°C (Grant Instruments, Cambridge). After complete drying, 200µl of RIA buffer (0.05M TRIS +4mM EDTA, pH 7.5) were added to each tube, followed by vigorous homogenization, until a 'milky' suspension was obtained. 1ml of assay buffer was added to each tube, followed by vortex mixing. A 10 or 20µl aliquot was removed from the supernatants in each tube, for estimation of protein by the method of Lowry et al (374) - see appendix 5.

Tubes were centrifuged at 13,000g for 10 minutes (Eppendorf centrifuge) to ensure complete separation of supernatant from precipitated protein. An aliquot was taken from the supernatant from each tube,and appropriately diluted for cyclic GMP RIA; supernatant from each tube was stored at 4°C,in case samples were required for re-assay.

1.2. Cyclic GMP RIA

The assay was carried out under similar conditions for construction of RIA standard curves,as discussed previously (see page 148). Standards from 0.06 to 2.0p moles cyclic GMP were employed in the assay. 50µl of diluted sample were used in place of standards for measurement of cyclic GMP, one or two dilutions of each sample being assayed. Assays were conducted using duplicate samples; values showing greater than 5% variation between duplicates were discarded, and re-assayed.

Prolonged storage of diluted antibody at 4°C resulted in gradual reduction of zero binding level which, although not affecting the sensitivity of the assay adversely, could reduce precision if zero binding was lowered too far. Antibody was subsequently stored in frozen aliquots, which were thawed and diluted before use. Generally, no more than 60 assay tubes were processed in the one experiment (standards and samples in duplicate), standard curves being constructed for each assay performed.

The majority of the cyclic GMP assays used the immunoglobulin fraction of a specific and sensitive antiserum supplied by the Radiochemical Centre, and employed in their cyclic GMP RIA kit (333). Due to the limited amounts of this antibody towards the latter part of my studies,

antibodies A7 B2 and A10 B5, whose specificity for cyclic GMP had been already established (see page 161), were also employed for a number of assays.

Dilution of assay samples was chosen to measure cyclic GMP in the range of 0.1 - 1.0p moles, where the standard curve has maximum slope and therefore greater precision (212), although detection limits (calculated as two standard deviations about zero binding) were calculated at approximately 0.03p mole in one experiment; the Radiochemical Centre cyclic GMP kit has a detection limit of 0.04p mole.

The desired detection limit of an assay is dependent upon the tissue levels of the substance to be measured. The high levels of cyclic GMP in cerebellum did not require exquisitely sensitive techniques for measurement, however. It should be considered though, that the higher dilution of a sample used in an assay, the less possibility there is of interference from cross-reacting molecules.

1.3. Comparison of tissue levels of cyclic GMP measured with different antibodies.

An experiment was carried out to determine the levels of cyclic GMP in incubated brain slices, using the immunoglobulin fractions of 'Amersham' antibody, A7 B2, and A10 B5, and also the cyclic GMP RIA kit. The data is shown in table 14, and demonstrates that similar values were obtained with the different antibodies.

1.4. Relation between sample dilution for RIA and cyclic GMP level.

Table 15 shows the measurement of cyclic GMP (p moles/mg protein) using different dilutions of RIA sample, to demonstrate the close agreement between cyclic GMP level at differing dilutions of assay sample, measured using A7 B2 and A10 B5 antibodies.

Table 14 Comparison of tissue levels of cyclic GMP measured with different antibodies.

Values expressed as p moles cyclic GMP/mg protein

TISSUE	AMERSHAM RIA 'KIT'	AMERSHAM IMMUNOGLOBULIN	A7 B2	A10 B5
THREE INDIVIDUAL INCUBATED 350μm RAT CEREBELLAR SLICES i)	18.9	18.9	19.2	19.9
ii)	27.8	-	28.1	28.8
iii)	20.0	21.3	18.4	16.8
INCUBATED 750μm MOUSE CEREBELLAR SLICE	-	-	44.3	45.4

Table 15 Relation between sample dilution for RIA and cyclic GMP level, using A10 B5 and A7 B2 antibodies.

Values expressed as p moles cyclic GMP/mg protein

DILUTION OF SAMPLE IN RIA	A10 B5	A7 B2
1/48	45.0	45.0
1/72	43.6	40.5
1/144	48.6	46.8
1/288	46.8	45.0
1/576	43.2	-

1.5. To determine whether the substance being measured by RIA is cyclic GMP.

Cross-reactivity data (see page 161) has shown that the antibodies employed for RIA are highly specific for cyclic GMP, showing minimal cross-reactivity with cyclic AMP, ATP and GTP, substances which might interfere in cyclic GMP tissue RIA. It is essential however, to demonstrate that specific removal of cyclic GMP from tissue extracts for assay, completely removes immuno-assayable material. There is a possibility that an unknown substance in the tissue extract could displace $[^3H]$cyclic GMP from antibody, and therefore be falsely measured in the RIA.

Cyclic GMP levels were therefore measured under normal and elevated conditions, in tissue extracts with, and without, the enzyme cyclic 3',5'-nucleotide phosphodiesterase. A small piece of cerebellum from a rat injected with either harmaline, a drug known to elevate cerebellar cyclic GMP levels (see page 241 for experimental details), or saline control, was extracted with ethanol and homogenized in an Eppendorf tube with 200μl of 1mM Imidazole/1.6mM Mg SO_4 buffer, pH 7.5. After complete homogenization, 1ml of this buffer was added to the tubes, which were then vortex mixed. 20μl of the homogenate were removed for the estimation of protein by the method of Lowry et al (see appendix 5). After 10 minutes centrifugation at 13,000g (Eppendorf centrifuge), an aliquot of the supernatant was taken from each tube and appropriately diluted with buffer ('harmaline' sample 1/144, 'saline' sample 1/24). 200μl of each diluted sample were incubated with 10μl of buffer containing 0.05 units** of phosphodiesterase (Sigma Chemical Co), whilst a further 200μl of each sample were incubated as control, with 10μl of buffer. Incubations were

** Unit definition: one unit will convert 10^{-6} moles of cyclic AMP to 5' AMP per minute at pH 7.5, at 30°C, with no significant associated enzyme activities.

carried out for one hour in Eppendorf tubes at 37°C in a water bath (Grant Instruments, Cambridge). Following the incubation, all samples were transferred to glass test tubes and heated for 10 minutes in a water bath at 100°C, to destroy the enzyme. Two 50µl aliquots from each tube were then assayed for cyclic GMP.

Table 16 shows that phosphodiesterase has completely destroyed measurable cyclic GMP.

Note: The discrepancy between the values in this table and table 17, showing the effect of harmaline on cerebellar cyclic GMP levels, may be due to samples being homogenized in the Imidazole/Mg SO_4 buffer (to optimize phosphodiesterase activity), but assayed in TRIS/EDTA RIA buffer.

Additional support for the immuno-assayable compound being cyclic GMP comes from studies with incubated brain slices, where inhibition of cyclic 3',5'-nucleotide phosphodiesterase with the compound IBMX, produced large increases in measurable cyclic GMP (see table 24)

Table 16 Effect of phosphodiesterase treatment on extracted cerebellar tissue samples, from two rats injected with saline control or harmaline, 30 minutes prior to sacrifice.

	SAMPLE	SAMPLE + 0.05 UNITS PHOSPHODIESTERASE
HARMALINE 40mg/Kg i.p.	22.6	Not detectable
SALINE CONTROL i.p.	16.3	Not detectable

Values expressed as p moles cyclic GMP/mg protein

1.6. Tissue deproteinization techniques for recovery of cyclic GMP.

The most common tissue deproteinization techniques employ perchloric and trichloroacetic acids or aqueous ethanol, to precipitate protein and prevent further metabolism of cyclic nucleotides (333). The ethanol technique however,is simple,and has the advantage that it may be used to concentrate very low levels of substances for assay.

An experiment was carried out to compare the recovery of endogenous cyclic GMP from rat cerebellum, using ethanol and trichloroacetic acid extraction. A small piece of cerebellum was removed from a rat, washed in ice-cold physiological saline, and divided into two equal parts.

Ethanol precipitation of protein:

One part of the tissue was thoroughly homogenized with 200µl of 80% ethanol-water in an Eppendorf tube. 1ml of the aqueous ethanol was then added and the tube was vortex mixed. After one hour, the contents of the tube were evaporated to dryness overnight at 60°C on a 'Dri-Block'. When thoroughly dry, the sample was homogenized with 200µl of RIA buffer, followed by addition of a further 1ml of buffer. After vortex mixing, a 20µl sample was removed from the homogenate for assay of protein. The tube was centrifuged for 10 minutes at 13,000g (Eppendorf centrifuge), an aliquot of the resulting supernatant being removed,and appropriately diluted for assay.

Trichloroacetic acid (TCA) precipitation of protein:

The second part of the tissue sample was thoroughly homogenized by a colleague at the same time as the 'ethanol' sample, in 200µl of'ice-cold' 7% TCA in an Eppendorf tube. A further 1ml of TCA was added,followed by vortex mixing;the tube was then centrifuged for 15 minutes at 13,000g (Eppendorf centrifuge). The supernatant was removed to a 10ml glass test tube, and washed four times with three volumes of water-saturated ethyl ether,

to remove all traces of TCA. The contents of the tube were then dried under a stream of nitrogen gas from a cylinder. 1200 µl of assay buffer were then added to the tube followed by shaking to completely dissolve the contents. An aliquot of the solution was removed and appropriately diluted for assay. The precipitate obtained from the centrifugation of the Eppendorf tube was dissolved in 1200 µl of 1N NaOH, and 20 µl were removed for assay of protein.

Results:

Tissue cyclic GMP-ethanol extraction	——	10.8p moles cyclic GMP/mg protein
Tissue cyclic GMP-TCA extraction	——	9.0p moles cyclic GMP/mg protein

Ethanol has also been shown to be more effective for cyclic AMP extraction, in comparison with TCA and perchloric acid (292). This is probably since the four wash-steps with ether are not required.

To check the recovery of exogenous cyclic GMP, 50 µl of [^{3}H] cyclic GMP were added to a small piece of cerebellar tissue. Following homogenization, evaporation, and reconstitution with buffer, 93% of the tracer was recovered.

2. ATTEMPTS TO CORRELATE CHANGES IN CYCLIC GMP 'IN VIVO' WITH IMMUNOHISTOCHEMICAL CHANGES.

2.1. Introduction.

Experiments were carried out to determine whether immunohistochemistry could localize drug-induced changes in cyclic GMP to specific cell types or loci within cells, in rat cerebellum.

It was initially important to ensure that cyclic GMP levels measured by direct homogenization of tissue samples after death, were equivalent to those in tissue that was frozen in the isopentane-liquid nitrogen mixture, in preparation for tissue sectioning for immunohistochemistry;

the possibility existed that freezing might in itself artefactually change cyclic GMP levels.

Experiments were carried out to measure cyclic GMP levels in tissue homogenized or rapidly frozen, 2 minutes after decapitation. Cerebellar samples were compared from rats either injected with the tremorogenic drug harmaline (40mg/Kg injected intra-peritoneally at 0.5mg/ml, using a 25 guage needle, 30 minutes before sacrifice), or physiological saline as control. After 5 minutes, rats injected with harmaline displayed fine tremor which was maintained up to the time of sacrifice. This drug was chosen, since it has been reported to cause large biochemical changes in cerebellar cyclic GMP, independent of cyclic AMP, by a well-defined mechanism - activation of the climbing fibre input to cerebellum (172, 173). After 30 minutes, rats were decapitated and part of the cerebellum was removed. Tissue was briefly washed in 'ice-cold' physiological saline and divided into two parts. One piece was homogenized in 80% ethanol/water, as described previously, whilst the other part was placed on a piece of labelled aluminium foil, which was attached to a metal frame and plunged into the isopentane-liquid nitrogen freezing mixture. With the assistance of a colleague, both these operations were carried out exactly 2 minutes after decapitation.

The sample homogenized directly in aqueous ethanol was assayed for cyclic GMP and protein, as described previously. After 1 minute in the freezing mixture, the other sample was transferred to the cryostat ($-25^{\circ}C$), and allowed to equilibrate for 30 minutes before being placed in a pre-cooled Eppendorf tube within the cryostat. 200μl of pre-cooled 80% ethanol/water were then added, ensuring that the tissue sample was covered. The tube was then removed from the cryostat and rapidly

homogenized, followed by addition of 1ml of aqueous ethanol and vortex mixing. Cyclic GMP and protein were then assayed, as described previously.

Table 17 shows that both direct homogenization, and freezing in isopentane-liquid nitrogen prior to homogenization gave similar levels of cyclic GMP, and confirm the elevation of cerebellar cyclic GMP levels by the drug harmaline (172, 173).

Table 17: Cerebellar cyclic GMP levels after saline or harmaline (40mg/Kg)injection 30 minutes prior to sacrifice - effect of direct homogenization,or freezing prior to homogenization.

Values expressed as p moles cyclic GMP/mg protein, from individual rat cerebella.

TREATMENT	DIRECT HOMOGENIZATION	FREEZING PRIOR TO HOMOGENIZATION
SALINE	6.1	6.0
	5.5	6.3
	8.8	Mean = 6.2
	Mean = 6.8	
HARMALINE 40mg/Kg i.p.	27.0	44.1
	36.0	18.5
	34.1	39.6
	25.5	25.7
	Mean = 30.7	Mean = 32.0

2.2. Cyclic GMP immunohistochemistry applied to cerebella from rats injected with harmaline, to elevate cyclic GMP levels.

6μm frozen sections were cut from 'saline' or 'harmaline' injected cerebella, and processed for cyclic GMP immunohistochemistry using three antibodies; Steiner G17 12/2/74, A7 B2 and A10 B5. Dilutions were chosen such that fluorescence with 'saline' injected sections was bright, but not maximal, in order to maximize any increases in intensity which might be observed with sections from 'harmaline' cerebella, as a result of increased cyclic GMP levels. 'Saline' and 'harmaline' sections were examined on the same slide to eliminate any differences in slide processing. Positive staining was observed with the same distribution, and the same intensity, in 'saline' and 'harmaline' sections by visual comparison. For a more objective comparison, photographs were taken from different areas of 'saline' and 'harmaline' sections, using the same exposure times and identical development and printing conditions, (see figure 49).

Comparison of prints taken from the molecular layer, granule layer and white matter areas of 'saline' and 'harmaline' sections showed no differences in distribution and/or intensity of cyclic GMP immunofluorescence; micro-dissection of mouse cerebella, however, had shown increased cyclic GMP biochemically, in the molecular layer, after harmaline injection (169).

These experiments have been carried out on a total of 8 rats injected with harmaline, and 8 saline-injected controls with identical results; they have also been independently confirmed by Y Koide of Dr Steiner's group (personal communication). Identical results were also obtained with 'saline' and 'harmaline' sections fixed by immersion in 'ice-cold'

Figure 49 Immunofluorescent localization of cyclic GMP in sections of cerebellum taken from rats injected with saline control or harmaline prior to decapitation, and the effect of buffer washing or fixation on immunofluorescence, prior to application of antibody.

All sections were processed identically using Steiner G17 7/15/74 cyclic GMP antibody.

a) Unfixed section from rat injected with saline control
b) Unfixed section from rat injected with harmaline } Calibration bar=50μm

c) Unfixed section from saline-injected rat, washed in buffer, prior to application of antibody
d) Unfixed section from harmaline-injected rat, washed in buffer, prior to application of antibody } Calibration bar=100μm

e) Section from saline-injected rat fixed in 'ice-cold' 2% freshly depolymerized paraformaldehyde and washed in buffer, prior to application of antibody
f) Section from harmaline-injected rat fixed in 'ice-cold' 2% freshly depolymerized paraformaldehyde and washed in buffer, prior to application of antibody } Calibration bar=50μm

M - molecular layer, P - Purkinje cell body, G - granule layer, W - white matter

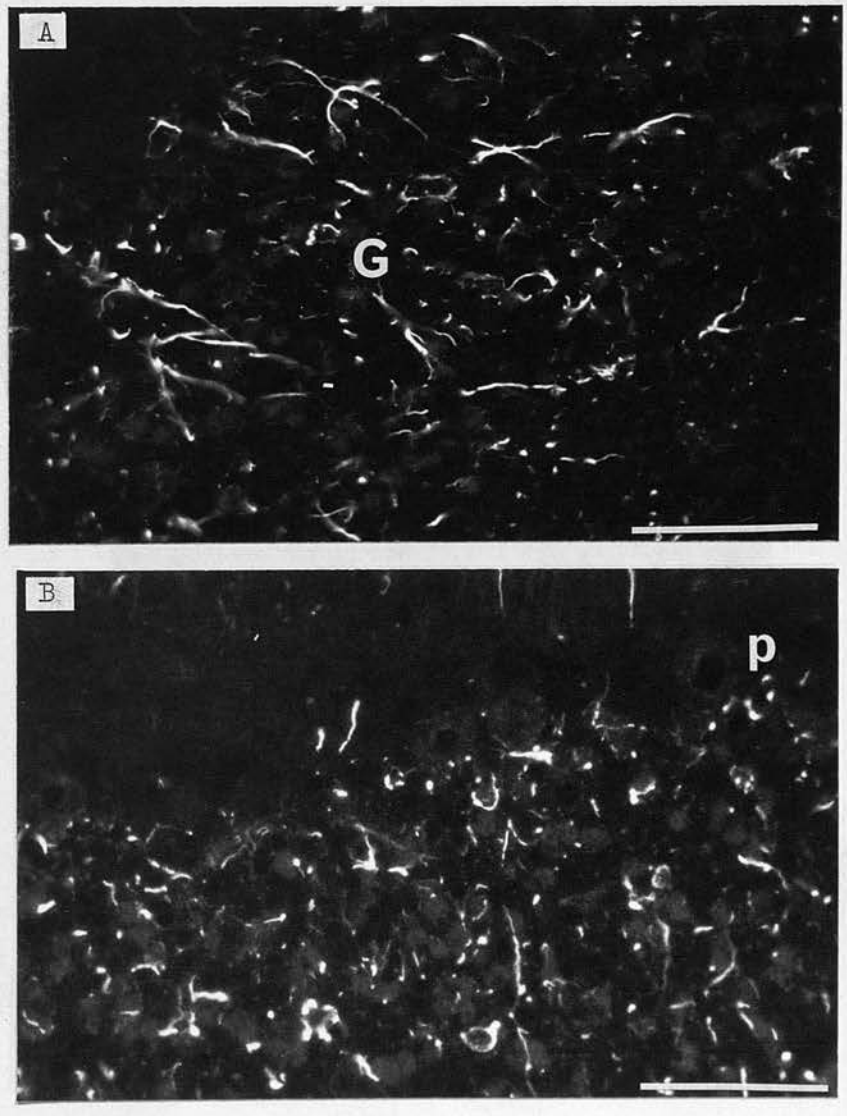
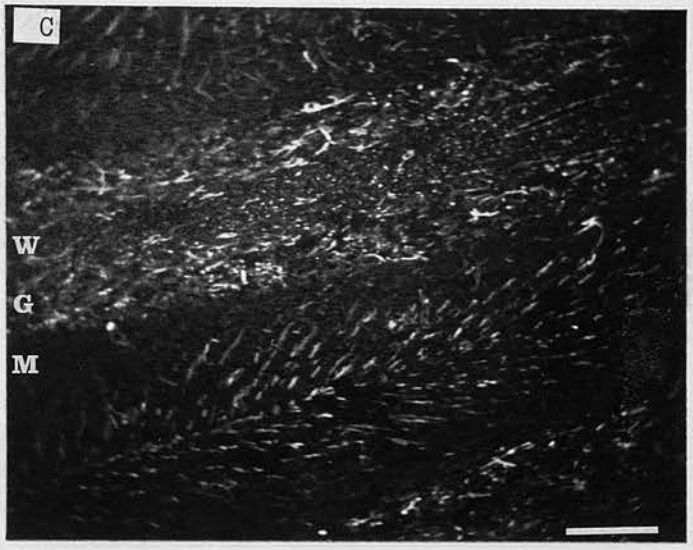


Figure 49 (Continued)



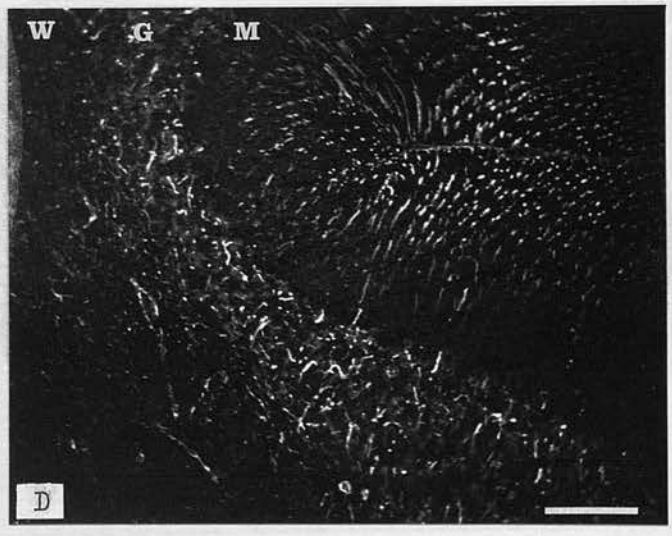


Figure 49 (Continued)

2% freshly depolymerized paraformaldehyde (see appendix 3), followed by 3 x 3 minute washes in PBS, prior to processing for immunofluorescence (see figure 49).

2.3. Cyclic GMP immunohistochemistry applied to cerebella from rats treated with either pentobarbitone or ethanol, to decrease cyclic GMP levels.

Whilst harmaline elevates cerebellar cyclic GMP levels several-fold, cyclic GMP levels have been reported to be decreased by a number of drugs which act as C.N.S. 'depressants' (55) e.g. intra-peritoneal injection of pentobarbitone has been shown to decrease cerebellar cyclic GMP levels (334), whilst intra-gastric intubation of rats with ethanol causes a dose-dependent decrease in cyclic GMP, in rat cerebellum (335).

Two groups of 3 rats were injected intra-peritoneally with either 40mg/Kg of pentobarbitone ('Sagital'-May & Baker Ltd, Dagenham, Essex) in physiological saline, or saline control. 5 minutes after injection, rats were decapitated, followed by freezing of cerebella in isopentane-liquid nitrogen, 2 minutes later. 6μm sections were processed for cyclic GMP immunohistochemistry and photographed, as before. No differences were found between intensity and/or distribution of specific fluorescence.

Ethanol was administered to a group of 4 rats by intra-gastric intubation as a 20% w.v. solution in water (335), water being given by the same method to 2 control animals. Rats were fasted prior to the experiment in order to clear their stomachs. By biting onto a clamped wooden stick drilled with a small hole, a narrow plastic tube, coated in liquid paraffin, was passed through the hole and fed into the stomach. It was important to ensure that the tube entered the oesophagus and not the trachea, passing completely into the stomach with minimum resistance.

Figure 50 Immunofluorescent localization of cyclic GMP in cerebellar sections taken from rats treated with either ethanol or water.

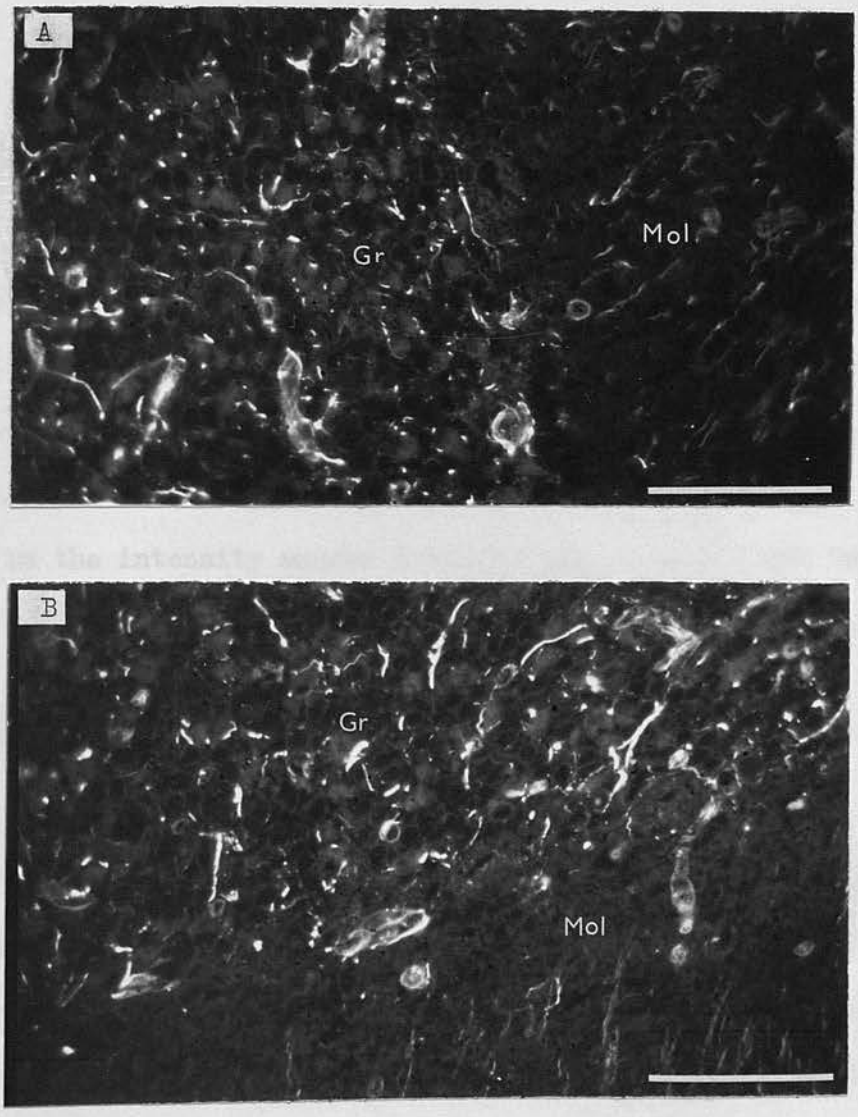
Sections were processed identically using Steiner G17 12/2/74 cyclic GMP antibody.

a) Cerebellar section from rat treated with ethanol solution by intra-gastric intubation.

b) Cerebellar section from rat treated with water (control) by intra-gastric intubation.

Calibration bar = 50μm

Gr - granule layer
Mol - molecular layer



Between 6 and 7ml of aqueous ethanol or water were slowly administered to each rat by means of a plastic tube and syringe.

Rats given aqueous ethanol became very drowsy on being returned to their cages, remaining sedentary until sacrifice one hour later; the greatest decrease in cerebellar cyclic GMP levels (335) has been found to occur at this time, after oral administration of ethanol. After decapitation the cerebella were removed and frozen 2 minutes later. 6µm frozen sections from 'control' or 'ethanol' cerebella showed no difference in intensity and/or distribution of specific cyclic GMP staining with Steiner G17 12/2/74 antibody, on visual or photographic comparison (see figure 50).

2.4. Conclusion.

Several drug treatments have been selected, which cause the most significant increases or decreases reported in cerebellar cyclic GMP levels 'in vivo'. Employing visual and photographic semi-quantitative techniques on unfixed and fixed frozen sections, no changes have been found in the intensity and/or distribution of cyclic GMP immunofluorescence with the three positive staining cyclic GMP antibodies examined.

3. ATTEMPTS TO CORRELATE CHANGES IN CEREBELLAR CYCLIC GMP 'IN VITRO' WITH IMMUNOHISTOCHEMICAL CHANGES

3.1. Introduction

Since attempts to correlate 'in vivo' biochemical changes with immunohistochemical changes had been unsuccessful, it was decided to employ 'in vitro' techniques to attempt to localize, in particular, the large biochemical changes in cyclic GMP induced by depolarizing agents, as reported by Ferrendelli's group (149, 150, 151).

Incubated respiring brain slices containing a heterogeneous cell population, have the advantage over 'in vivo' studies that the extra-cellular environment of the tissue may be precisely defined and controlled, although they are not subject to effects that may occur in the intact brain. To illustrate this latter point, harmaline which is effective in elevating cerebellar cyclic GMP levels several-fold 'in vivo' causes a slight decrease in levels 'in vitro' (169). This is because it exerts its effect 'in vivo' by stimulating the intact climbing fibre input to the cerebellum, thereby indirectly elevating cerebellar cyclic GMP levels (172, 173).

Preliminary biochemical studies were carried out in incubated cerebellar slices to establish that significant elevations in cyclic GMP could be obtained by a number of agents, acting by different mechanisms. A method was developed for freezing incubated slices and cutting frozen sections, which could then be processed in the usual way for cyclic GMP immunohistochemistry. This technique had previously been used by Kebabian et al (239) for cyclic nucleotide localization in incubated slices of bovine superior cervical ganglion, and Kapoor and Krishna (336) for pancreatic slices, but represented the first application to 'in vitro' studies with C.N.S. tissue.

3.2. Preparation and processing of incubated cerebellar slices.

A Krebs-Ringer incubation buffer was continually gassed with 95% O_2,5%CO_2 (British Oxygen Co. Ltd, London) from a cylinder, for 1 hour 'on-ice', and used for preparation of tissue slices,as decribed below. The constituents of the buffer were: 120mM NaCl, 4.7mM KCl, 25mM $NaHCO_3$, 1.2mM KH_2PO_4, 2.4mM $MgSO_4$, 2mM $CaCl_2$ and 10mM glucose (149).

Rats were stunned with a sharp blow, and decapitated; the cerebella and

underlying brain stem were removed. Mice were killed by cervical dislocation. Care was taken to ensure that cerebella were not damaged during decapitation or during removal from the skull; cerebella were discarded if damaged in any way. Rat or mouse tissue was briefly washed in 'ice-cold' buffer, and transferred to a small piece of filter paper soaked in buffer, and resting on an 'ice-cold' Petri dish. Tissue from the brain stem was carefully removed, and the cerebellum was positioned on a drop of glycerol on a small filter paper circle, in a McIlwain tissue chopper (Mickle Laboratories, Surrey; see reference 307). The cerebellum was orientated so that coronal sections could be cut. A small amount of glycerol was also placed on the sharp razor blade of the tissue chopper, and slices were then cut at either 350μm or 750μm, at maximum cutting rate. The chopped tissue and filter paper were then immersed in 'ice-cold' buffer and slices were carefully teased apart with the aid of forceps. Possibly a more rigorous selection procedure was used for slices than would normally be employed by other workers in biochemical experiments, since immunohistochemistry was also to be performed on sections taken from these slices; rarely, therefore, were more than three or four slices obtained from each cerebellum. Selected slices were transferred to a vial containing fresh buffer which was sealed, and stored on ice. The procedure was repeated as rapidly as possible with remaining animals, slices being stored in the sealed vial. When all the slices had been obtained, they were washed several times with fresh buffer and transferred to 60ml of 'ice-cold' pre-gassed buffer in the pre-incubation vessel. This was incubated in a water bath (Grant Instruments, Cambridge) at 37°C, pre-incubation time commencing when the buffer attained this temperature.

In early experiments, slices were incubated with buffer in a beaker, the medium being replenished with fresh, pre-gassed buffer at 37°C, every 30 minutes. Comparison of this technique with one employing continuous gassing of the medium, revealed much lower levels than with the continuous gassing technique, however. This was probably due to inadequate oxygenation of incubated slices, and continuous gassing was therefore used for all future experiments.

A length of polypropylene tubing sealed at one end was fixed down the side, and to the bottom, of a glass beaker where it formed a circle. Passage of gas down the tube and into the buffer through perforations in the walls of the tube enabled the medium to be continuously gassed, the bubbles gently agitating the incubated slices. At the end of the pre-incubation period, slices were lifted onto a metal holder and each rapidly transferred to a similar continuous gassing apparatus, for incubation with the test agent. After incubation, slices were individually removed using the metal holder, and rapidly homogenized in 200µl of 80% ethanol-water, in Eppendorf tubes. 1ml of the aqueous ethanol was added, vortex mixed, and samples were assayed for cyclic GMP and protein.

For immunohistochemistry, slices were placed on labelled cork mats in a metal frame and rapidly frozen in the isopentane-liquid nitrogen freezing mixture. After equilibration in the cryostat, 6µm sections were cut and processed for cyclic GMP immunohistochemistry. With the help of a colleague, slices could be homogenized or frozen at the same time after removal from the incubation medium; this period was standardized at 30 seconds.

3.3. Experiments using incubated slices of rat cerebellum.

A preliminary experiment demonstrated that incubated slices would have to be at least 750μm thick to enable sections to be cut for immunohistochemistry, after freezing. 750μm rat cerebellar slices were pre-incubated for two hours, the period reported for stabilization of basal levels after the post-mortem decapitation increase in cyclic nucleotides (337). Cyclic GMP levels were then measured after additional incubation for 15 minutes in fresh Krebs-Ringer buffer, and after 15 minutes incubation with a depolarizing agent-'high potassium' Krebs-Ringer buffer. This buffer, as described in reference 149, substituted the 4.7mM KCl in the normal buffer with 120 mM KCl. To maintain the overall ionic strength of the buffer, the NaCl concentration was reduced from 120mM (normal buffer) to 5mM in the 'high potassium' buffer. The results shown in table 18 demonstrate the appreciable variation in basal cyclic GMP levels in the rat cerebellar slices, and levels after 'high potassium' buffer. Two slices assayed from the same rat showed significant variation in basal levels, suggesting that the cause was not inter-animal variation. It was considered that the variation might be a result of the slice thickness used. 'In vitro' biochemical studies are generally carried out on slices not greater than 400μm, since this is the theoretical maximum for adequate oxygenation of the interior of a C.N.S. slice (307).

Experiments were repeated using 350μm incubated slices. Table 18 shows that similar results were obtained with wide variation in cyclic GMP levels between slices, even from the same animal, and lack of stimulation with 'high potassium' buffer, indicating that the effect was not related to inadequate oxygenation. This variability and lack of stimulation, precluded the use of rat cerebellar slices for immunohistochemical studies.

Table 18 Cyclic GMP levels in individual rat cerebellar slices of 750μm and 350μm thickness, after incubation for 15 minutes in normal Krebs-Ringer or 'high potassium'(120mM) buffer.

2 hour pre-incubation in normal Krebs-Ringer buffer. Values expressed as p moles cyclic GMP/mg protein.

	15 minute incubation in normal Krebs-Ringer buffer	15 minute incubation in 'high potassium' Krebs-Ringer buffer
750μm THICKNESS	26.0 21.2 28.4 14.2 34.8) Rat 1 22.9)	30.0 15.2 30.2 36.5 38.0 Rat 1
350μm THICKNESS	28.0 10.3 14.7 20.8 30.4) Rat 2 26.4) 16.8) Rat 3 28.8)	10.8 8.7 8.6 11.7 12.6) 21.6) Rat 2 33.3) 24.2) Rat 3 19.9)

These results differ from those of Schmidt et al (337) who showed that after two hours pre-incubation, 'high potassium' buffer caused a significant elevation in cyclic GMP levels in rat cerebellum. These workers used finely chopped tissue rather than uniform slices, however, which may account for the stimulation they reported. Kinscherf et al (149), although showing higher cyclic GMP levels than I have reported in these studies, have also demonstrated extreme variability in basal levels in machine cut 300-350 μm slices from rat cerebellum, and small stimulation with 'high potassium' buffer, as compared with cerebellar slices from a number of other species.

Incubation of adult rat cerebellar slices has been found to result in swelling and pyknosis of neural and glial cells by ultrastructural techniques (153). Tissue which has been hand-cut however, shows better preservation than that which has been machine-chopped as employed in these studies. The biochemical levels of cyclic GMP in hand-chopped rat cerebellar tissue have been found to be constant under basal conditions, and to show large responses to depolarizing stimuli (personal communication, Dr J Garthwaite).

3.4. Experiments using incubated slices of mouse cerebellum.

In contrast to the variable basal levels of cyclic GMP in rat cerebellar slices, incubated slices of adult mouse cerebellum at 750 μm and 350 μm, showed relatively constant basal levels, after one hour pre-incubation, similar to those reported by Ferrendelli's group (149, 150, 151). Incubation for five minutes with 'high potassium' buffer elevated cyclic GMP levels approximately 5-fold in slices of both thicknesses (see table 19). This increase, whilst significant, is considerably less than that reported by Ferrendelli et al (150,151), who demonstrated maximal increases of approximately 40-fold with

Table 19 Cyclic GMP levels (p moles/mg protein) in individual 750μm and 350μm mouse cerebellar slices, after incubation in normal Krebs-Ringer or 'high potassium' (120mM) buffer.
Bracketed values represent slices processed in same experiment. (1 hour pre-incubation in normal Krebs-Ringer buffer).

Significant difference between incubation in normal and 'high potassium' Krebs-Ringer buffer - $p<0.01$, Wilcoxan two-tailed rank test.

	Basal cyclic GMP levels after pre-incubation	5 minute incubation in 'high potassium' Krebs-Ringer buffer
750μm THICKNESS	6.2 4.6 4.3 6.3 6.0 3.6 3.1 4.3 6.2 3.1 6.0 4.6 Mean = 4.9 Standard deviation = 1.24	32.8 21.6 24.5 29.6 26.0 Mean = 26.9 Standard deviation = 4.38
350μm THICKNESS	7.0 8.8 4.8 4.8 4.5 6.4 4.4 4.4 5.1 8.6 Mean = 5.9 Standard deviation = 1.72	25.9 26.2 28.8 27.9 35.5 43.2 21.6 Mean = 29.9 Standard deviation = 7.21

respect to basal levels after five minute stimulation in this buffer. Whilst differences in experimental technique might be partly responsible for the variation, strain differences between mice may be significant. This has been suggested by Saito (155), studying responses of cyclic GMP in incubated slices exposed to adenosine.

Increasing the incubation time in high potassium buffer above five minutes however, resulted in more variable responses between slices.

To ensure that the stimulation of cyclic GMP was a result of the changed potassium concentration of the buffer, cyclic GMP levels were measured in 750μm mouse slices after one hour pre-incubation in normal Krebs-Ringer buffer, and in slices transferred and incubated for either 5, 10, 15 or 20 minutes in buffer of identical composition. The results in table 20 show that the process of transferring slices had no significant effect on cyclic GMP levels, and that basal levels were constant for at least 80 minutes of pre-incubation.

Table 20 Cyclic GMP levels (p moles/mg protein) in individual 750μm mouse cerebellar slices transferred after incubation, and incubated in identical buffer for different periods of time.

BASAL CYCLIC GMP LEVELS AFTER PRE-INCUBATION	MINUTES OF INCUBATION IN IDENTICAL BUFFER			
	5	10	15	20
5.3	8.5	7.5	4.0	6.6
6.7				
6.8				

120mM KCl was employed as 'high potassium' buffer, since this concentration was found by Ferrendelli et al (150, 151) to give maximal elevation of cyclic GMP in mouse cerebellar slices.

In one experiment 750μm slices were incubated in 'high potassium' buffer at 20mM and 60mM KCl; NaCl concentration was altered accordingly to maintain ionic strength. The results shown in table 21 indicate that whilst 20mM KCl only caused a slight elevation in cyclic GMP levels, 60mM KCl produced similar results to incubation in 120mM KCl 'high potassium' buffer (compare with table 19).

Table 21 Cyclic GMP levels (p moles/mg protein) in individual 750μm mouse cerebellar slices, after 5 minutes incubation in 20mM KCl or 60mM KCl.
(n = number of slices)
see table 19

Basal cyclic GMP levels after pre-incubation	20mM KCl	60mM KCl
Mean = 4.9 (n = 12) Standard deviation = 1.24	11.8 8.8 6.8 6.9	24.0 42.0 27.9 28.8 25.0 36.6
	Mean = 8.6 Standard deviation = 2.34	Mean = 30.7 Standard deviation = 7.09

The dependency of 'high potassium' stimulation of cyclic GMP on calcium ions, as previously reported (150, 151), was confirmed by pre-incubating 750μm mouse cerebellar slices in Krebs-Ringer buffer for 45 minutes, followed by incubation for 30 minutes in buffer without calcium ions, and with the addition of 1mM EGTA. Three slices were then incubated for either 5, 10 or 15 minutes in 120mM 'high potassium' buffer without calcium ions, and with the addition of the chelating agent; elevation of cyclic GMP was blocked under these conditions (see table 22 below).

Table 22 Cyclic GMP levels (p moles/mg protein) in individual 750μm mouse cerebellar slices, after incubation with 'high potassium' (120mM) buffer, in the absence of calcium ions.

Basal cyclic GMP levels after 45 minutes pre-incubation	Cyclic GMP levels after 30 minutes incubation in calcium-free Krebs-Ringer + 1mM EGTA	Minutes of incubation in 'high potassium' (120 mM) buffer - calcium + 1mM EGTA		
		5	10	15
5.7	4.7 5.6	4.0	9.0	9.0

To determine whether inhibition of cyclic nucleotide phosphodiesterase would significantly elevate cyclic GMP levels in mouse cerebellar slices, after 1 hour pre-incubation in normal Krebs-Ringer buffer, three slices were incubated for either 5, 10 or 15 minutes in buffer containing 1mM 3-isobutyl 1-methyl xanthine (IBMX, Aldrich, 'Gold Label'), a potent inhibitor of phosphodiesterase. The results show that cyclic GMP levels were elevated with respect to control (see table 23).

In the same experiment, three slices were incubated after pre-incubation, for either 5, 10 or 15 minutes in 'high potassium' buffer + 1mM IBMX. After 5 minutes incubation, the cyclic GMP level was elevated approximately 10-fold compared with control. These results, shown in table 23, suggest that 'high potassium' buffer elevates cyclic GMP due to an action on the synthesizing enzyme, rather than by inhibiting phophodiesterase.

Table 23 Cyclic GMP levels (p moles/mg protein)in individual 750µm mouse cerebellar slices incubated in normal Krebs-Ringer or 'high potassium' (120mM) buffer, in the presence of a phosphodiesterase inhibitor. (n = number of slices) see table 19

Basal cyclic GMP levels after pre-incubation	Minutes of incubation in Krebs-Ringer buffer + 1mM IBMX			Minutes of incubation in 'high potassium' buffer + 1mM IBMX		
	5	10	15	5	10	15
Mean = 4.9 (n = 12)	18.1	14.3	15.0	54.3	48.8	46.6

In a further experiment, slices were pre-incubated for 1 hour in normal Krebs-Ringer buffer containing 1mM IBMX; greater than 16-fold elevation in cyclic GMP levels was observed, compared with slices pre-incubated in normal buffer without the phosphodiesterase inhibitor. Subsequent incubation of three slices with'high potassium' buffer, produced very large increases in cyclic GMP levels, demonstrating the capacity of the incubated mouse cerebellar slice for accumulating cyclic GMP (see table 24).

The calcium-dependent effect of cyclic GMP stimulation with 'high potassium' buffer was presumably due to an indirect effect on the soluble guanylate cyclase, which requires calcium ions for activation (114).

Table 24 Cyclic GMP levels (p moles/mg protein) in individual 750μm mouse cerebellar slices after 1 hour pre-incubation in normal Krebs-Ringer or Krebs-Ringer with addition of 1mM IBMX, followed by incubation with 'high potassium' (120mM) buffer + 1mM IBMX.
(n = number of slices)
see table 19

Basal cyclic GMP levels after pre-incubation in normal Krebs-Ringer buffer	Cyclic GMP levels after pre-incubation in Krebs-Ringer + 1mM IBMX buffer	Time in minutes after incubation in 'high potassium' buffer + 1mM IBMX		
		5	7½	20
Mean = 4.9 (n = 12)	90.4 71.3 78.4	150.9	192.0	117.3

The particulate guanylate cyclase which is present in mouse cerebellum (338), is calcium-independent, and has been shown to be stimulated by sodium azide and other agents, elevating cyclic GMP levels in incubated slices of rat cerebellum and other tissues (339). To demonstrate whether similar elevations could be elicited in slices of mouse cerebellum, 750μm slices were pre-incubated for 1 hour in normal Krebs-Ringer buffer, followed by incubation with 1mM sodium azide.

Cyclic GMP levels were elevated to a similar extent as for incubation with 'high potassium' buffer (table 25). In a similar experiment to that described previously (see table 22), sodium azide was found to elevate cyclic GMP in the absence of calcium ions, presumably due to activation of the calcium-independent guanylate cyclase.

The effect of the neurotransmitter noradrenaline was also investigated on mouse cerebellar slices, since it has been found to elevate cyclic GMP levels in a glial cell line (165). Noradrenaline (L-ARTERENOL HCL,

Table 25 Cyclic GMP levels (p moles/mg protein) in individual 750μm mouse cerebellar slices incubated with 1mM sodium azide in the presence or absence of calcium ions.

	Minutes of incubation in sodium azide ± calcium ions				
	5	7½	10	12½	15
1mM sodium azide + 2mM Ca (normal Krebs-Ringer buffer)	14.8	31.5	25.7	29.6	30.3
1mM sodium azide - Ca + 1mM EGTA	22.5 32.8	24.9	31.3	29.3	30.9 48.5

Table 26 Cyclic GMP levels (p moles/mg protein) in individual 750μm mouse cerebellar slices incubated with 1mM noradrenaline.

Basal cyclic GMP levels after pre-incubation in normal Krebs-Ringer buffer	Minutes of incubation in 1mM noradrenaline		
	5	10	15
5.7 4.7 5.6	10.9	12.7	3.5

Sigma Chemical Co) was dissolved in buffer directly prior to the start of the experiment since oxidation rapidly occured, producing a pink coloration of the medium.

A small increase was observed in cyclic GMP levels after incubation with the neurotransmitter (see table 26).

3.5. Cyclic GMP immunohistochemistry applied to mouse cerebellar slices.

Mouse cerebellar slices were incubated under identical conditions to those used for biochemical estimations, for subsequent immunohistochemical processing. Slices were incubated with the following agents:

1) 7 slices were pre-incubated for 1 hour in normal Krebs-Ringer buffer as control.

2) 4 slices were pre-incubated for 1 hour in normal Krebs-Ringer buffer, followed by 5 minutes incubation in 'high potassium' (120mM) buffer.

3) 3 slices were pre-incubated for 1 hour in normal Krebs-Ringer buffer, followed by 5 minutes incubation in 1mM sodium azide.

4) 2 slices were pre-incubated for 1 hour in normal Krebs-Ringer buffer, followed by 5 minutes incubation in 1mM noradrenaline.

5) 4 slices were incubated for 1 hour in Krebs-Ringer buffer containing 1mM IBMX.

The process of freezing incubated slices was found to have no effect on cyclic GMP levels in comparison with biochemical assay after direct homogenization, observations comparable with those found for 'in vivo' studies (see table 17).

Although incubated slices were prepared at 750µm, some difficulty was experienced in cutting serial 6µm frozen sections for immunofluorescence. Sometimes only small parts of sections could be obtained due to the frozen tissue slice being 'wrinkled'. Sections were only processed if visual examination of the frozen section clearly showed a number of cerebellar folia.

Although 750µm and 350µm incubated slices responded in a similar way biochemically, it was possible that part of the slice might have been unresponsive due to dead cells or swelling of cells, and that cyclic GMP might be unevenly distributed through the slice. The outer layer of cut cells would, in particular, be damaged during the long incubation procedure. Frozen sections were therefore cut from a number of the incubated slices at different positions through the slice, to compensate for any variation.

Cyclic GMP immunohistochemistry was performed on either unfixed frozen sections, or those fixed with 2% freshly depolymerized paraformaldehyde prior to application of the cyclic GMP antibody. A10 B5 and Steiner G17 antibodies were both used, but as stated previously (see table 11) only bleed 12/2/74 of the latter antibody showed positive staining, and fibre staining was absent.

Visual and photographic semi-quantitative techniques were unable to show any differences in distribution and/or intensity of specific staining, in any of the cell layers with any of the treatments, or between sections from the same slice (see figures 51 and 52). Experiments were repeated several times, using antibody dilutions designed to give minimal specific fluorescence under basal conditions, in an attempt to maximise any differences between basal and stimulated slices.

Figure 51 Immunofluorescent localization of cyclic GMP in frozen sections, from mouse cerebellar slices incubated with 120mM 'high potassium' buffer or 1mM sodium azide, or under control conditions.

Sections processed identically with A10 B5 cyclic GMP antibody.

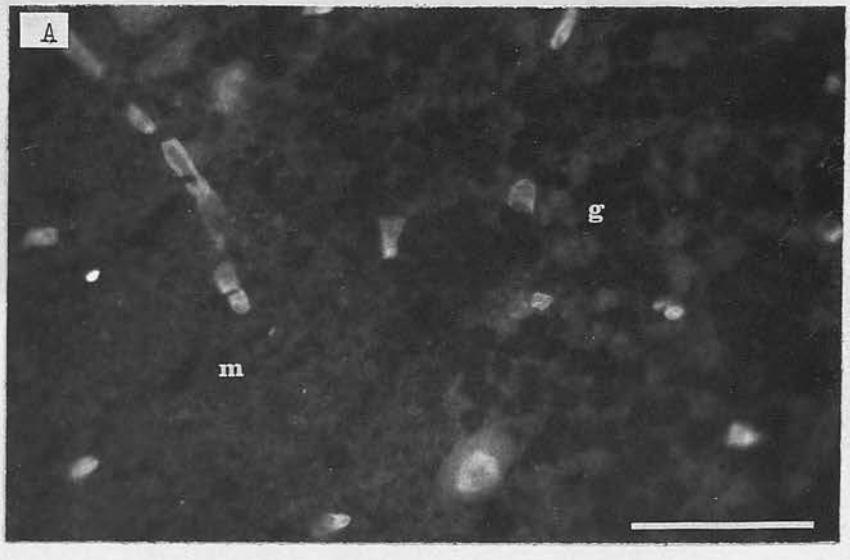
a) Slice incubated in normal Krebs-Ringer buffer.

b) Slice incubated in 120mM 'high potassium' buffer.

c) Slice incubated in Krebs-Ringer buffer containing 1mM sodium azide.

Calibration bar = 50 μm

g - granule layer
m - molecular layer



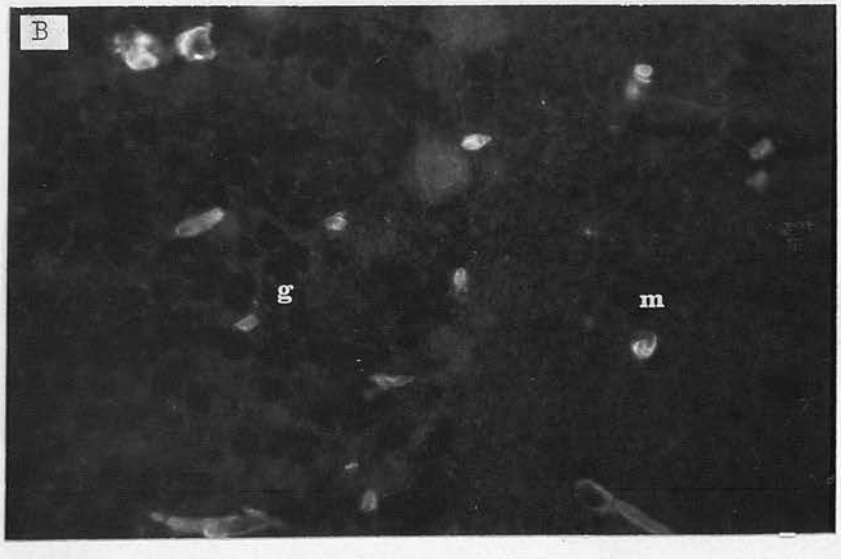


Figure 51 (Continued)

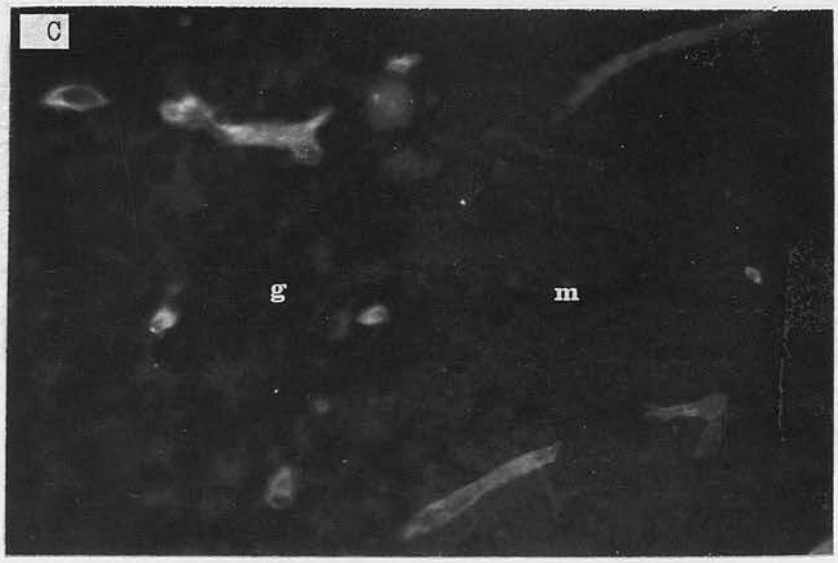


Figure 52 Immunofluorescent localization of cyclic GMP in frozen sections from mouse cerebellar slices, incubated in the presence or absence of 1mM noradrenaline.

Sections processed using Steiner G17 antibody (bleeds 7/15/74 and 12/2/74).

a) Slice incubated in normal Krebs-Ringer buffer; section incubated with Steiner G17 antibody(bleed 7/15/74).

b) Slice incubated in normal Krebs-Ringer buffer; serial section to that used in a) above, incubated with Steiner G17 antibody (bleed 12/2/74).

c) Slice incubated in normal Krebs-Ringer buffer; section incubated with Steiner G17 antibody (bleed 12/2/74). High magnification.

d) Slice incubated in Krebs-Ringer buffer containing 1mM noradrenaline; section incubated with Steiner G17 antibody (12/2/74). High magnification.

Calibration bar for a) and b) = 100µm

Calibration bar for c) and d) = 50µm

G - granule layer
M - molecular layer
P - Purkinje cell body layer

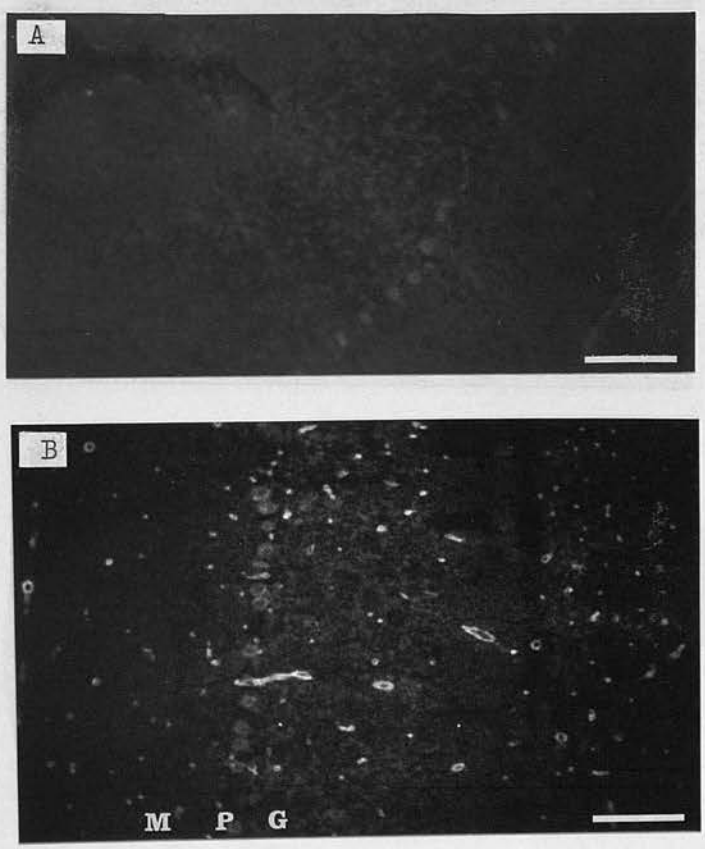
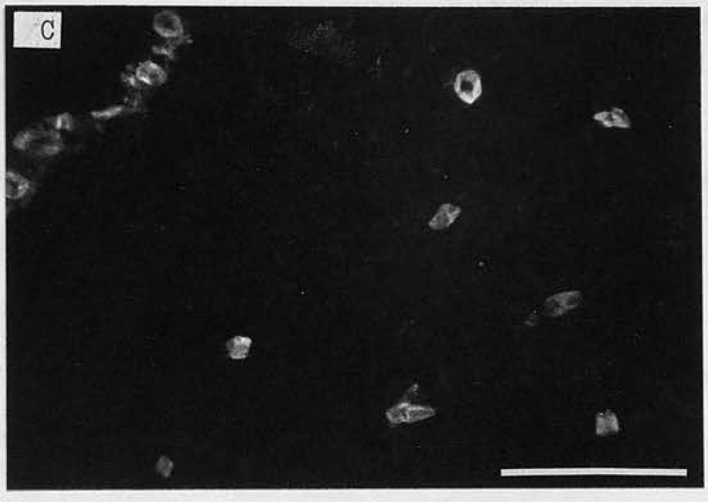
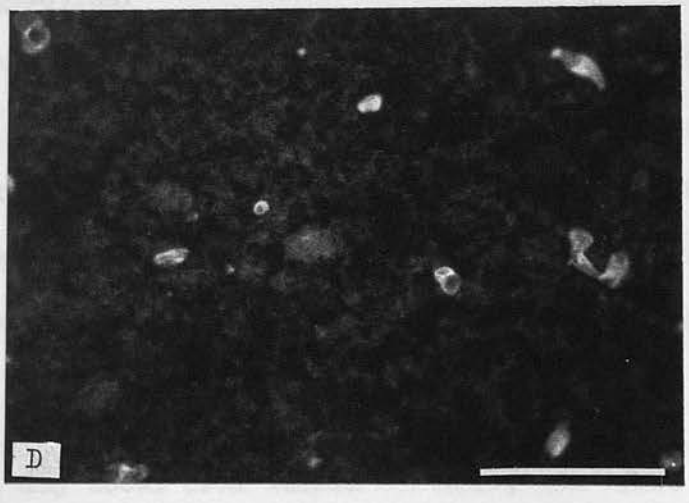


Figure 52 (Continued)





3.6. Conclusion.

Using both 'in vivo' and 'in vitro' techniques, no changes have been observed in intensity and/or distribution of cyclic GMP immunofluorescence, under a variety of conditions in which cyclic GMP tissue levels have been shown to significantly change biochemically.

The absence of correlation suggests that immunofluorescence might not be localizing 'stimulated' cyclic GMP, which might be lost on the tissue sections during immunohistochemical processing, by either being soluble or loosely bound to the tissue; the experiments in the following section were designed to examine this possibility.

4. CYCLIC NUCLEOTIDE LOSSES FROM CEREBELLAR TISSUE SECTIONS DURING PROCESSING FOR IMMUNOHISTOCHEMISTRY.

4.1. Introduction.

In histochemical studies examining losses of enzymes from tissue sections, Wachsmuth (340) has shown that whilst membrane-bound enzymes are not lost from unfixed buffer-washed tissue sections, soluble enzymes e.g. aldolase and lactate dehydrogenase, show significant losses from unfixed or acetone-fixed sections, on subsequent immersion in buffer.

Steiner et al (215) have suggested that losses of the low molecular cyclic nucleotides may occur from tissue sections, and could be quantified by measuring cyclic nucleotides extracted from tissue sections, in the buffer washings. It seems likely that the reason why losses have not been measured experimentally by groups using cyclic nucleotide immunohistochemistry, is that increases in fluorescence have paralleled the biochemical increase in levels in a number of tissues (e.g. see references 236 and 239).

In experiments designed to measure cyclic GMP losses from rat cerebellar frozen sections, quantification in buffer washings was not attempted since levels would be extremely low, almost certainly prohibiting measurement. Cyclic GMP levels were therefore measured in frozen sections, before and after buffer-washings. Unwashed sections contain total nucleotide i.e. bound and free cyclic GMP, whilst washed sections will contain only that pool of cyclic GMP which is tissue - bound i.e. the amount remaining, and therefore potentially able to be visualized, on the tissue section.

Measurement of tissue levels of cyclic nucleotides in homogenates, are obtained by extraction of the nucleotides after protein denaturation. The levels obtained are total levels and do not give any information on whether the nucleotides are free or bound i.e. to soluble or tissue-bound specific receptor proteins. Recently, the occupation of cyclic AMP-dependent kinase receptor protein, by cyclic AMP, has been measured under different conditions to estimate free and bound nucleotide (341). In the following experiments which are described, free and bound refer to whether cyclic nucleotides are attached to insoluble tissue proteins; the bound pool is that potentially available for immunohistochemical localization, since the possibility exists that 'hidden' antigenic determinants on the tissue-bound nucleotide, could prevent access of total nucleotide in the bound pool, to specific antibody.

4.2. Cyclic GMP losses from buffer-washed cerebellar sections taken from controlled rats.

To determine the effect of buffer washing on tissue cyclic GMP, unfixed sections were assayed for cyclic GMP and protein, and serial sections were washed and agitated for 3 x 3 minutes in buffer prior to assay, to remove soluble or loosely bound nucleotide.

Preliminary experiments showed that approximately 30 x 10μm frozen sections would be required to accurately determine both protein and cyclic GMP levels, using the assay procedures already described. A total of 60 x 10μm frozen sections were cut from each of 10 cerebella frozen 2 minutes after decapitation, at different positions throught the cerebella, to minimize any possible regional differences in cyclic GMP. Alternate sections were placed on two coated glass slides (appendix 1); the 30 sections were placed close together on each slide. After drying the slides in air for 30 minutes, one slide was labelled 'unwashed' and remained on the bench, whilst the other was placed in a metal staining rack, and washed three times for 3 minutes in 400ml of PBS in a staining dish. On removal from the dish, both slides were dried under cool air from a hair-dryer, to evaporate buffer. Initial experiments showed that distilled water could be substituted for PBS as the third wash, without affecting biochemical measurements, or immunofluorescence; this prevented the white film of buffer salts forming on the 'washed' slide, after drying.

For estimation of cyclic GMP and protein levels, sections were carefully scraped off each slide with a plastic pipette tip and 80% aqueous ethanol, and washed into Eppendorf tubes with a total of 1.2ml of the aqueous ethanol. The tubes were vortex mixed, and allowed to stand for 1 hour. After this time they were opened and placed in a 'Dri-Block' (Grant Instruments, Cambridge) overnight, at 60^{o}C. When dry, the contents of the tubes were homogenized in either 120μl or 150μl of radioimmunoassay buffer (0.05M TRIS/4mM EDTA), followed by centrifugation at 13,000g for 10 minutes (Eppendorf centrifuge). Duplicate 50μl samples were used directly in the RIA for measurement of cyclic GMP. The remaining supernatants were removed, and the precipitates re-suspended in 50μl

of distilled water. Aliquots of the suspension were appropriately diluted, duplicate samples of 20μl being used for estimation of protein.

The results shown in table 27 demonstrate the very large loss (greater that 80%) of cyclic GMP, after buffer-washing of tissue sections. The values are expressed as p moles cyclic GMP/mg protein, to correct for any variation in tissue quantity taken from the assay. Washing, however, causes a loss of soluble proteins to the buffer. If the results are expressed as p moles cyclic GMP in washed and unwashed sections, the percentage loss is even greater than shown in the table. This is important when comparing fluorescence in washed and unwashed tissue sections, since intensity is dependent upon quantity of cyclic GMP, rather than concentration.

The large losses in cyclic GMP show that in cerebella taken from rats and frozen 2 minutes after decapitation, only a small fraction of cyclic GMP remains on the tissue section after buffer washing. Processing tissue during immunohistochemistry requires either 6 or 7 buffer washes altogether, so it could be possible that the fraction of cyclic GMP contributing to the fluorescent staining pattern could be even less than reported here, where three buffer washes have been employed. However, employing overnight washing of tissue sections in gently agitated PBS, prior to application of specific antibody, no significant difference was found in staining compared with sections processed after the standard procedure; this suggests that the tissue-bound pool visualized by immunofluorescence is constant after short buffer washing.

It is possible to estimate the amount of cyclic GMP potentially available for immunofluorescent staining in each section, from these results.

Table 27 Cyclic GMP levels (p moles/mg protein) in pooled 10μm rat cerebellar sections either unwashed, or after 3 x 3 minute PBS buffer washes.

Rat number	Unwashed sections	Washed sections	% loss from unwashed sections
1	2.30	0.30	87.0
2	2.69	0.25	90.7
3	3.23	0.22	93.2
4	2.73	0.39	85.7
5	2.34	0.28	88.0
6	2.66	0.58	78.2
7	1.68	0.26	84.5
8	2.02	0.40	80.2
9	2.57	0.27	89.5
10	1.92	0.38	80.2
	Mean = 2.41	Mean = 0.33	Mean = 85.7
	Standard deviation = 0.46	Standard deviation = 0.11	Standard deviation = 4.95

Cyclic GMP levels in washed sections significantly different from unwashed sections, $p<0.01$ Wilcoxan matched-pairs two-tailed rank test.

Table 27 shows that a mean value of 0.33 p moles cyclic GMP/mg protein is bound on tissue sections after washing. Since 30 x 10μm cerebellar sections contain approximately 0.7mg protein, it can be calculated that one 10μm section contains approximately 7.7×10^{-15} moles of cyclic GMP.

4.3. Cyclic GMP losses from buffer-washed cerebellar sections taken from rats injected with either harmaline or saline control.

To determine whether changes occurred in soluble or tissue-bound cyclic GMP, after elevation of levels induced by drugs, an experiment was carried out to determine the effect of prior administration of the drug harmaline on cyclic GMP levels in unwashed or washed rat cerebellar sections.

Two groups of 7 rats were injected intra-peritoneally with either saline control or harmaline (40mg/Kg), injected 30 minutes prior to sacrifice; cerebella were frozen 2 minutes after decapitation.

The results shown in table 28 demonstrate that whilst harmaline elevated cyclic GMP levels approximately 3-fold in unwashed sections, it had no effect on levels in washed sections, with respect to saline-injected control. The results may be interpreted as showing that whilst harmaline elevates total cyclic GMP levels in cerebellum, the stimulated pool is either soluble or loosely bound to the tissue, and therefore lost by buffer washing, whilst the bound pool remains constant.

The process of sectioning and thawing tissue sections may account for the lower levels of cyclic GMP in frozen unwashed sections, as compared with cerebella similarly frozen, but homogenized and then assayed. To determine which factor was responsible, frozen sections might be pooled and extracted with ethanol in the cryostat at -25°C, to prevent thawing of sections at room temperature.

Table 28 Cyclic GMP levels (p moles/mg protein) in pooled 10µm rat cerebellar sections either unwashed or after 3 x 3 minute buffer washes, from rats injected with either saline control or harmaline, 30 minutes prior to sacrifice.

SALINE Control		HARMALINE 40mg/Kg	
UNWASHED	WASHED	UNWASHED	WASHED
1.99	0.45	5.69	0.60
1.60	0.37	4.59	0.49
2.29	0.67	2.13	0.41
1.82	0.58	5.37	0.59
1.70	0.34	5.70	0.47
1.50	0.29	6.33	0.38
1.15	0.26	7.75	0.52
Mean = 1.72	Mean = 0.42	Mean = 5.37	Mean = 0.49
Standard deviation = 0.36	Standard deviation = 0.16	Standard deviation = 1.73	Standard deviation = 0.08

Each pair of results (washed and unwashed) were obtained from the same animal; 7 rats were used in each group.

Significant difference, $p<0.01$ between saline unwashed and harmaline unwashed sections. No significant difference at the 5% level between saline washed and harmaline washed sections; Wilcoxan unpaired two-tailed rank tests.

Since no difference was observed in the intensity and/or distribution of cyclic GMP immunofluorescence, in sections taken from mouse cerebellar slices pre-incubated with a phosphodiesterase inhibitor prior to freezing, compared to control (see page 263), if phosphodiesterase becomes active on thawing of sections to room temperature, it has no effect on cyclic GMP immunofluorescence. The similar staining patterns of cyclic nucleotide immunofluorescence at different times of antibody incubation (see page 185 and reference 215), suggest that phosphodiesterase is not active when processing air-dried tissue sections.

Wachsmuth (340) has demonstrated that loss of the soluble enzyme aldolase from buffer-washed tissue sections, can be prevented during immunofluorescence by the aldolase antibody cross-linking the antigen. To test the possibility that cyclic GMP antibody might prevent cyclic GMP losses, tissue sections were either washed for 3 x 3 minutes in PBS prior to application of the cyclic GMP antibody, or processed as usual without this preliminary washing. No differences in distribution and/or intensity of specific staining were found. Figure 49 indicates that antibody did not prevent losses of either basal or stimulated cyclic GMP, as localized by immunofluorescence.

4.4. Cyclic AMP losses from buffer-washed sections taken from rat cerebella frozen within 30 seconds or at 2½ minutes after decapitation.

4.4.A. Confirmation of post-mortem elevation of cyclic AMP levels in tissue homogenates.

A small piece of cerebellum was taken from each of 5 rats frozen within 30 seconds or 2½ minutes after decapitation. After transfer from the isopentane-liquid nitrogen freezing mixture to the cryostat, and subsequent equilibration at -25°C, samples were homogenized in 80% aqueous ethanol in Eppendorf tubes, followed by evaporation overnight at 60°C.

Samples were assayed for cyclic AMP, using the assay kit supplied by the Radiochemical Centre, Amersham, Buckinghamshire (TRK 432; see also reference 342). The kit uses a binding protein instead of an antibody for measuring the competitive displacement of [^{3}H] cyclic AMP by unlabelled cyclic AMP. Protein-bound cyclic AMP is separated from free cyclic AMP using charcoal adsorption. Levels of cyclic AMP were expressed as p moles cyclic AMP/mg protein.

Table 29 Effect of post-mortem decapitation on rat cerebellar cyclic AMP (p moles/mg protein).

RAT 1	87.6	Cerebella frozen within 30 seconds after decapitation
RAT 2	69.6	
RAT 3	73.8	
RAT 4	181.1	Cerebella frozen at 2½ minutes after decapitation
RAT 5	218.4	

The results confirm the post-mortem increase in biochemical tissue levels of cyclic AMP in rat cerebellum; this increase has been localized histochemically, as increased numbers of Purkinje cells fluorescing (see figure 26 and reference 236).

4.4.B. Cyclic AMP losses from buffer-washed tissue sections.

In two experiments cyclic AMP was assayed in washed and unwashed 10μm sections taken from rat cerebella frozen within 30 seconds, or at 2½ minutes after decapitation. The methodology was similar to that described previously for the cyclic GMP studies.

Table 30 Cyclic AMP levels (p mole^{s}/mg protein) in pooled 10μm sections from rat cerebella frozen within 30 seconds or at 2½ minutes after decapitation; sections unwashed or after 3 x 3 minute buffer washes.

	CEREBELLA FROZEN WITHIN 30 SECONDS AFTER DECAPITATION			CEREBELLA FROZEN 2½MINS AFTER DECAPITATION		
	unwashed	washed	% loss from washed sections	unwashed	washed	% loss from washed sections
EXPERIMENT I	13.7	9.0	34.3	20.2	10.8	46.5
EXPERIMENT II	19.5	11.4	41.5	21.1	10.0	52.6

Individual values from pooled 30 x 10μm sections, from individual cerebella.

4.4.C. Discussion

The results from the small sample shown in table 30 (above), indicate that bound levels of cyclic AMP are not significantly different in washed sections from rat cerebella decapitated at two different times, whilst immunofluorescence can detect changes due to the post-mortem increase. Increased fluorescence may occur, therefore, with a very small increase in bound cyclic AMP that has not been detected with these experiments. Alternatively, a redistribution of the nucleotide may occur on stimulation, causing increased levels at certain sites and therefore increased fluorescence, without a net change in bound cyclic AMP levels. It is also interesting that a significant, though smaller loss of cyclic AMP compared to cyclic GMP, has been found from the unfixed rat cerebellar sections.

An alternative method has been used to quantify the fraction of cyclic AMP utilized for immunofluorescence (Dr T W Rall, personal communication). In these studies cyclic AMP levels in mouse cerebella frozen within 30 seconds after decapitation were 20-25 p moles/mg protein, as compared with 60-85 p moles/mg protein, 2½ minutes after decapitation. These values were obtained by homogenization and extraction of frozen tissue samples, and therefore represent total cyclic AMP. The amount of cyclic AMP extracted from unfixed frozen tissue sections however with cyclic AMP antibody, was approximately 2 p moles/mg protein from animals decapitated at both time intervals. Similar cyclic AMP immunofluorescent staining patterns were observed between rat and mouse cerebella, and increased numbers of mouse Purkinje cells were seen to show specific fluorescence in the studies reported by Dr Rall. These results confirm that only a small fraction of the cyclic nucleotide content measured biochemically, contributes to the immunofluorescent staining patterns.

5. GENERAL DISCUSSION

An important aim of this work was to determine whether immunohistochemistry could localize changes in biochemical tissue levels of cyclic nucleotides, to particular cellular sites. The cerebellum was studied in detail for these studies, since as discussed in the introductory literature review, it contains high levels of both cyclic AMP and cyclic GMP, which suggests that both nucleotides may have an important function in this brain area. In applying cyclic nucleotide immunohistochemistry to the C.N.S, Bloom et al (236) were able to localize cyclic AMP in the cerebellum and demonstrate increased numbers of Purkinje cells fluorescing, when biochemical levels were increased after post-mortem decapitation.

These observations have been repeated, demonstrating that for studies on cyclic AMP, immunohistochemistry may be a useful tool. However, the function of immunolocalizable cyclic AMP in other cellular elements must be considered. If a Purkinje cell pool is responsive to noradrenaline, perhaps the other pools might respond to stimulation by other neurotransmitters. Siggins et al (237) have tested the effect of 5 putatative neurotransmitters (noradrenaline, GABA, glycine, 5- HT and histamine) by topical application, and shown that none have significant effects on the pools of cyclic AMP that are not associated with the Purkinje cells. That immunohistochemistry may reveal different pools of cyclic AMP in a tissue stimulated under different conditions, is demonstrated in studies in the human peripheral lymphocyte (215):-

In lymphocytes incubated with prostaglandin E 1, all of the cyclic AMP specific fluorescence was localized to the cytoplasm, with no staining in the nucleus. Cells incubated with phytohemagglutinin, however, showed a 'patchy' fluorescence along the plasma membrane, whilst isoproterenol elicited staining throughout the entire cell.

Biochemical studies on cyclic GMP in the cerebellum have been carried out not only to determine levels in tissue homogenates, but also in an attempt to determine the cellular localization of the nucleotide. Micro-dissection of mouse cerebella coupled with highly sensitive radioimmunoassay (169), demonstrated that cyclic GMP was concentrated in the molecular layer of the cerebellar cortex and that a variety of drugs and conditions known to elevate cerebellar cyclic GMP, increased the concentration of the nucleotide in this layer.

Studies with mutant mice with Purkinje cell deficiency, have also suggested that a pool of cyclic GMP may be associated with this cell type (170, 171).

The cyclic GMP immunofluorescent studies reported in this chapter, however, have failed to correlate elevations in biochemical levels of the nucleotide with increased fluorescence in the positive staining structures (astrocytic fibres and capillaries), and it is important to note that fluorescence has neither been observed in Purkinje cells nor other neuronal elements.

Since biochemical changes in tissue levels as great as 16-fold have not been detected, whereas much smaller increases have been successfully localized by cyclic nucleotide immunofluorescence in other tissues (e.g. see references 239 and 336), the sensitivity of the technique would appear to be adequate. For precise measurement of fluorescence intensity rather than semi-quantification, micro-spectrophotofluorimetry coupled with interferometry, for measurement of section thickness, would be required (343).

The possibility exists that the reason why increases in staining intensity with respect to control have not been observed under conditions where tissue levels of cyclic GMP are elevated, is that under basal conditions, binding sites are fully saturated with the nucleotide. To test this possibility, immunohistochemistry has been applied to cerebellar sections taken from animals where drug administration (pentobarbitone, ethanol) has been reported to cause a decrease in tissue cyclic GMP levels. The results confirmed that the pool of cyclic GMP localized in tissue sections did not change under conditions where total cyclic GMP levels as measured biochemically were not only increased, but also decreased.

Development of a technique to determine whether immunohistochemistry localized all, or only a fraction, of cyclic GMP present in a frozen tissue section, demonstrated greater than 80% loss during buffer-washing for immunofluorescence. Similar experiments with cyclic AMP suggested a smaller, but significant loss, and demonstrated that total nucleotide was not being localized.

It was of interest that the fraction of cyclic GMP remaining in the tissue after washing and therefore potentially localizable, was unchanged when sections were taken from the cerebella of rats injected with harmaline, in order to elevate total cyclic GMP levels. These results may explain why changes in cyclic GMP immunofluorescence were not observed in tissues with elevated levels of cyclic GMP, since stimulated cyclic GMP might be in a soluble or loosely bound pool and therefore lost during processing.

It may be concluded, therefore, that cyclic GMP immunohistochemistry when applied to the C.N.S. is an unsuccessful technique for localizing biochemical changes, since only a fraction of the total nucleotide is localized on the tissue section.

CHAPTER VI

STUDIES ON THE BOUND POOL OF CYCLIC NUCLEOTIDES DETECTED BY IMMUNOFLUORESCENCE IN C.N.S. TISSUE SECTIONS

CHAPTER VI

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1. INTRODUCTION

Cyclic nucleotide immunohistochemistry is successfully performed on unfixed frozen tissue sections, in contrast to the localization of many other antigens which require fixation in order to be visualized (for example, see reference 311). Since specific receptor proteins are found in tissues which bind cyclic AMP and cyclic GMP, and are assumed to function as intermediates in eliciting the physiological responses attributed to these molecules (34), it is reasonable to assume that as the receptor proteins have both a soluble and particulate cellular distribution (181, 344, 345), they may bind the cyclic nucleotides in tissue sections.

Recently, antisera have become available to the cyclic nucleotide - dependent protein kinase holoenzymes, and their regulatory and catalytic sub-units. Steiner's group (247) have demonstrated that the receptor components may be localized in liver by immunofluorescence with these antisera, and that the histochemical distribution of these proteins may change under acute and chronic conditions.

In March 1978 I was able to visit Dr Steiner's laboratory for a second time, to determine whether the receptor components could be localized by immunofluorescence in the C.N.S.

2. CYCLIC GMP-DEPENDENT PROTEIN KINASE IMMUNOHISTOCHEMISTRY

In cerebellum, cyclic GMP kinase has been inferred biochemically to have a similar distribution to cyclic GMP, from experiments employing mutant mice and lesion studies of the cerebellar climbing fibre input (184); these results are compatible with cyclic GMP binding, and activating, a specific protein kinase, to mediate its effect in a Purkinje cell pool.

Cyclic GMP kinase holoenzyme, prepared to apparent homogeneity from bovine lung, by affinity chromatography with 8-(2-aminoethyl)-thio-cyclic GMP-Sepharose (330), was used to immunize rabbits by injection in Freund's adjuvant; specificity was checked by Dr Steiner's group using Ouchterlony immunodiffusion tests (247).

2.1. Rat C.N.S.

Immunofluorescence on cerebellar sections from 2 rats was performed using overnight incubation periods and equivalent immunoglobulin concentrations (0.05mg protein/ml) of Steiner GK#5 specific cyclic GMP kinase antibody, and the immunglobulin fraction of pre-immune serum from the same rabbit, to serve as control. Specific staining was found with a punctate appearance in the granule layer, and was uniformly distributed in the molecular layer, with white matter being almost completely devoid of staining (see figure 53). The nuclei and cytoplasm of occasional Purkinje cells showed positive staining. The fluorescent structures in the granule layer probably represent glomeruli, complexes of pre- and post-synaptic terminals between mossy fibres, granule cell dendrites and terminals of Golgi axons (346); a similar localization was found in the mouse cerebellum.

Extending the study to areas of the rat C.N.S. outside the cerebellum, very bright specific staining was observed between the mid-brain and the hemispheres, corresponding to the choroid fissure. Figure 54 shows a coronal section of rat brain at the level of the substantia nigra, demonstrating cyclic GMP kinase immunofluorescence in the fissure, presented in the form of a montage. Figure 55 shows in high magnification the plexus-like nature of the staining (which was not observed with cyclic GMP antibodies), and probably derives from the pia mater. See also figures 29 and 30.

Figure 53 Immunofluorescent localization of cyclic GMP-dependent protein kinase in rat and rabbit cerebellum.

a) Rat cerebellum - pre-immune immunoglobulin control.
b) Rat cerebellum - Steiner GK#5 cyclic GMP kinase antibody.

} Calibration bar = 50 μm

c) Rat cerebellum - Steiner GK#5 cyclic GMP kinase antibody. High magnification. Calibration bar = 25 μm.

d) Rabbit cerebellum - Steiner GK#5 cyclic GMP kinase antibody. Calibration bar = 50 μm.

g/G	-	granule layer
m/M	-	molecular layer
gl	-	glomerulus
P	-	Purkinje cell body
W	-	white matter

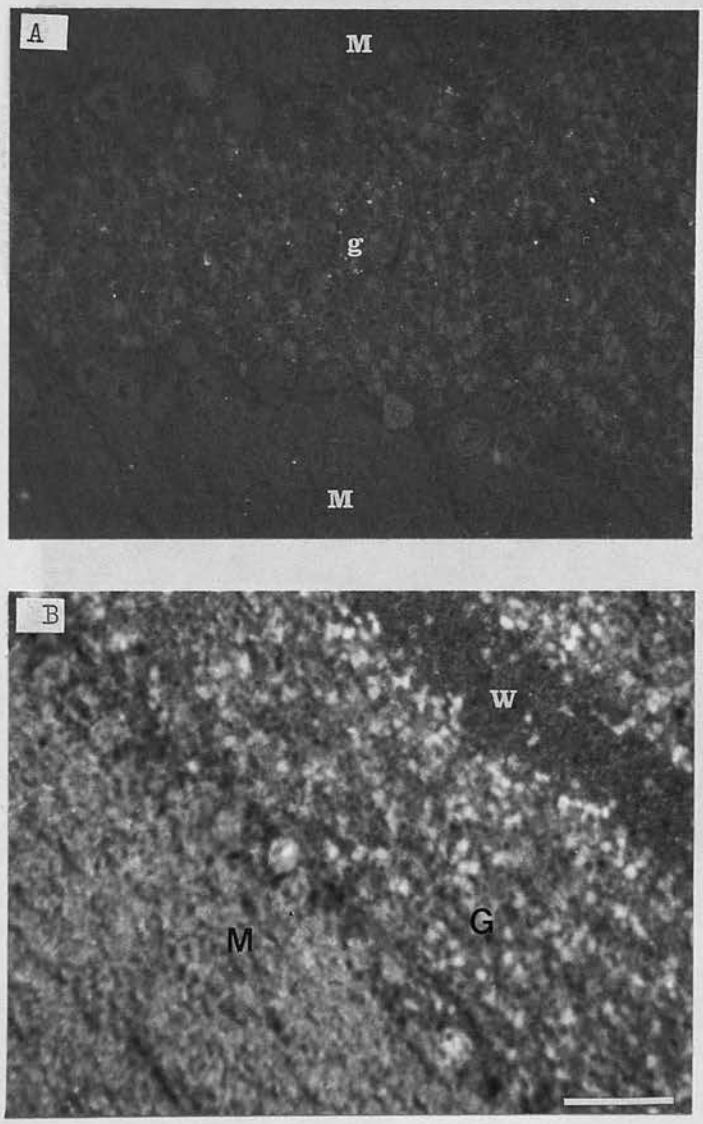
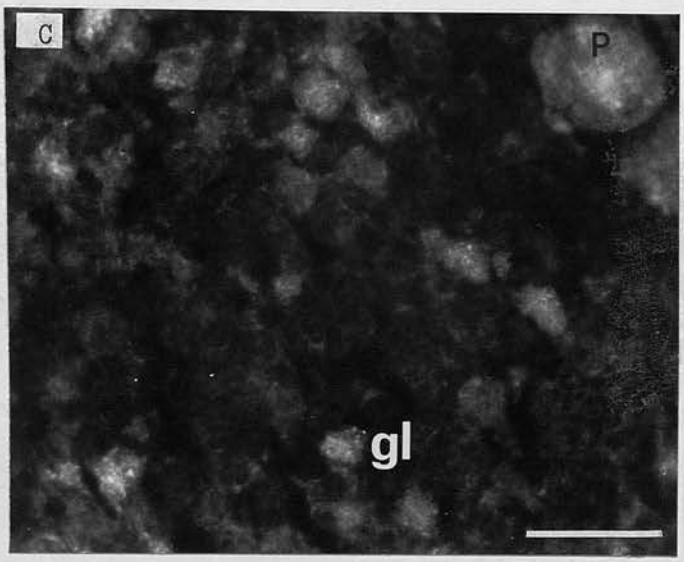


Figure 53 (Continued)



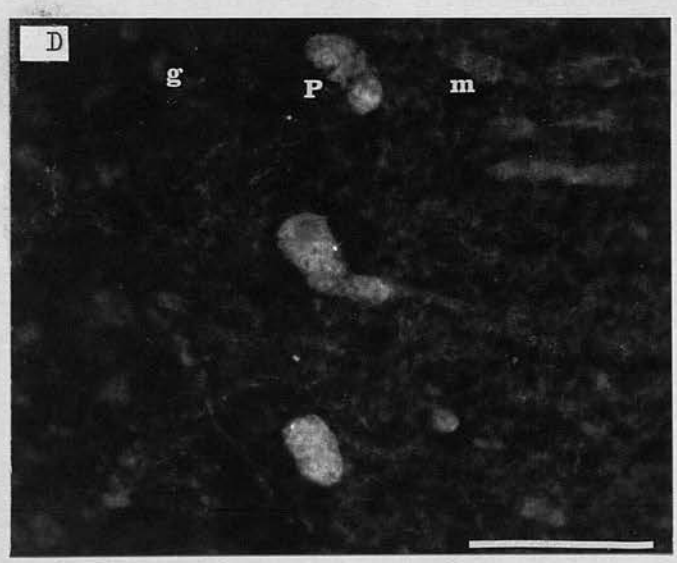


Figure 54 Photographic montage to show the immunofluorescent localization of cyclic GMP-dependent protein kinase in the choroid fissure of the rat brain, at the level of substantia nigra.

Steiner GK#5 cyclic GMP kinase antibody.

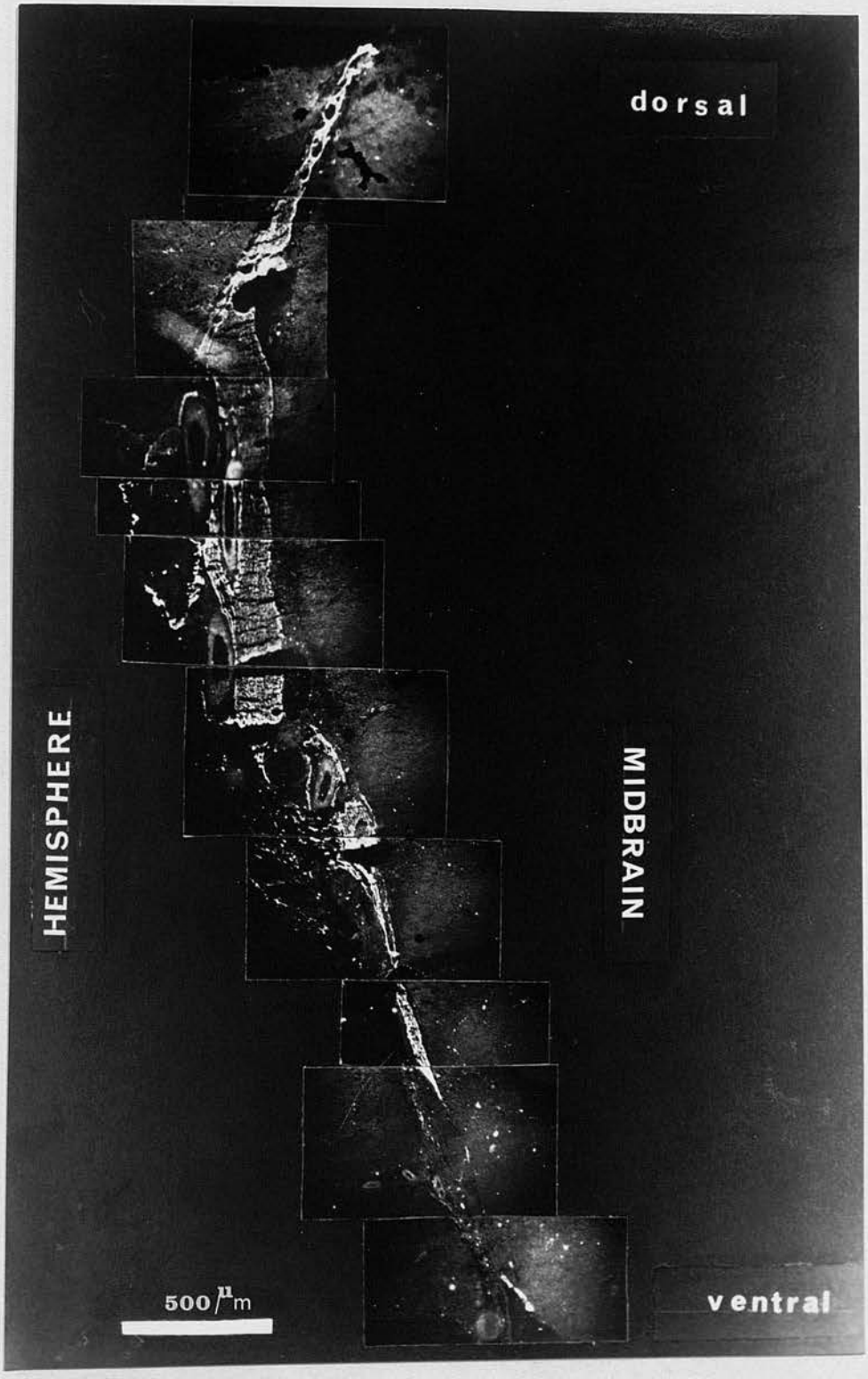


Figure 55 Immunofluorescent localization of cyclic GMP-dependent protein kinase in the choroid fissure of the rat brain, at the level of substantia nigra - high magnification.

a) Pre-immune immunoglobulin control.
b) Steiner GK#5 cyclic GMP kinase antibody.
} Calibration bar = 100 µm

White spots in a) represent artefacts.

2.2. Rabbit C.N.S.

Experiments were carried out to determine the immunofluorescent localization of cyclic GMP and cyclic GMP kinase in the cerebellum and spinal cord of the rabbit.

Two adult, New Zealand white rabbits, were stunned by a sharp blow and decapitated; the cerebellum was removed and a small portion of medial lobe was frozen in isopentane-liquid nitrogen freezing mixture within 5 - 10 minutes. Removal of a portion of lumbar spinal cord and freezing, was accomplished within 20 minutes of decapitation. 6μm frozen sections of rabbit tissue were subsequently processed for immunofluorescence.

The distribution of cyclic GMP immunofluorescence in rabbit cerebellum was similar to that observed in the rat (see figure 43), but with antibody to cyclic GMP kinase, bright specific staining was observed in the Purkinje cells, which was not found with pre-immune immunoglobulin (see figure 53).

In sections of spinal cord, cyclic GMP fibre staining was observed with a similar distribution to that reported for GFAP antigen (347), the fibre 'mesh' being clearly observed around unstained large neurones in the anterior horn (see figure 56). Once again, in contrast to the immunofluorescent localization of cyclic GMP, cyclic GMP kinase staining was observed in the cytoplasm of these large cells, with a 'speckled' appearance (see figure 57). Less intense staining was also observed in a number of smaller cells in both the grey and white matter of the cord.

It is interesting that a pool of cyclic GMP appears to be associated with large motor neurones of the spinal cord, from biochemical studies with normal mice and the 'wobbler' mutant (348).

Figure 56 Immunofluorescent localization of cyclic GMP-positive fibres in rabbit spinal cord.

Section processed with Steiner G17 7/15/74 antibody.

The crosses represent large unstained neurones in the grey matter of the cord.

Calibration bar = 50µm

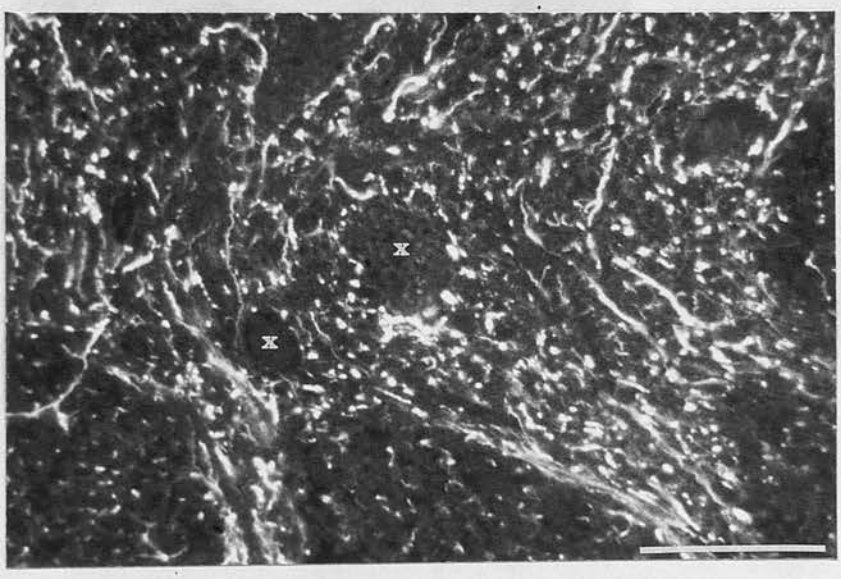


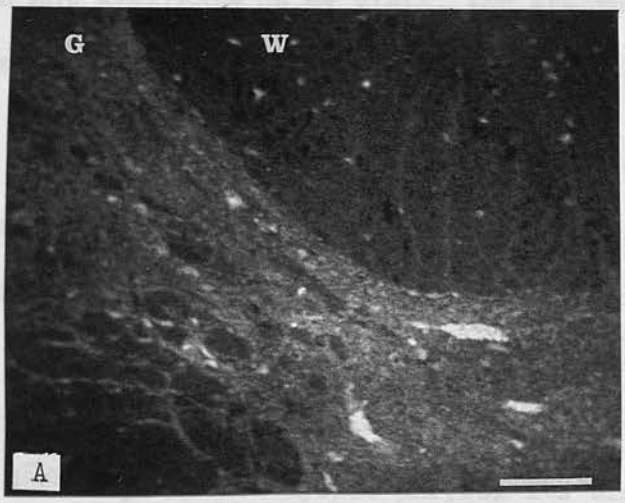
Figure 57 Immunofluorescent localization of cyclic GMP-dependent protein kinase in rabbit spinal cord.

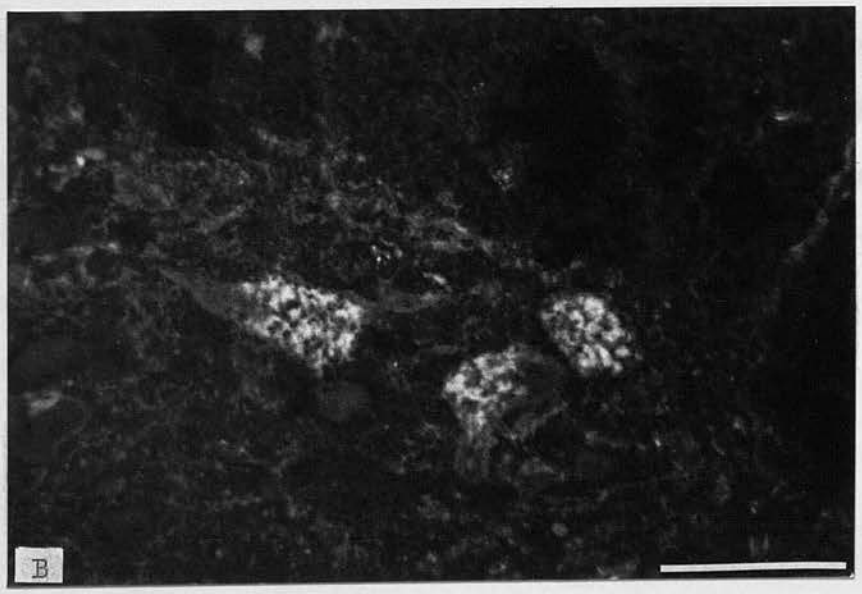
Steiner GK#5 cyclic GMP kinase antibody.

a) Low magnification. Calibration bar = 100 μm.

b) High magnification, showing brightly fluorescent cells in the grey matter. Calibration bar = 50 μm.

G - grey matter
W - white matter





2.3. Discussion

These preliminary results on the localization of cyclic GMP kinase, are of particular interest in showing that it does not parallel that of cyclic GMP, as observed by immunofluorescence. The specificity of Steiner GK#5 cyclic GMP kinase antibody has been demonstrated by Steiner's group using Ouchterlony immunodiffusion tests against related cyclic nucleotide receptor proteins, but unfortunately the antigen was not available on my visit, for absorption tests to demonstrate complete specificity of the C.N.S. staining patterns.

The strong staining of Purkinje cells in rabbit cerebellum may reflect increased amounts, or availibility to antibody, of the cyclic GMP kinase relative to the rat or mouse. The parallel localization of cyclic GMP kinase and cyclic AMP in the Purkinje cells by immunofluorescence, might represent a mechanism of cellular control, since cyclic AMP can compete with cyclic GMP,for its binding site on the kinase (349). Staining for cyclic GMP kinase in the choroid fissure is particularly surprising, and warrants further investigation.

An important theoretical consideration is that antibody to cyclic GMP kinase might not recognize a complex of cyclic GMP/cyclic GMP kinase in a tissue section, due to blockade of antigenic determinants. This possibility seems unlikely however, since parallel localization has been observed with the two molecules in rat liver (personal communication Dr Steiner).

3. CYCLIC AMP-DEPENDENT PROTEIN KINASE IMMUNOHISTOCHEMISTRY IN THE C.N.S.

3.1. Catalytic sub-unit.

The catalytic sub-unit of cyclic AMP-dependent protein kinase, prepared to homogeneity from rabbit skeletal muscle (350), has been used by Steiner's group to prepare specific antibodies, which have been employed for immunofluorescence. In liver for example (247), a reticular distribution in liver cell nuclei has been shown histochemically, which changes after acute drug treatment. Applying the catalytic unit antiserum at a dilution of 1:160, together with pre-immune serum at the same dilution (using overnight incubation periods), a striking distribution of specific staining was found in the nuclei of cells in rat cerebellum (figure 58). 'Liquid-phase' absorption of catalytic unit antiserum with excess catalytic unit overnight at 4°C (100µl of antiserum 1:1 dilution + 100µl of 0.8mg/ml of catalytic unit antigen), and subsequent dilution to give original antibody dilution (1:160), completely removed nuclear staining.

3.2. RII regulatory unit.

An antiserum raised in a goat to the RII regulatory unit of cyclic AMP-dependent protein kinase was also used for immunofluorescence in rat cerebellum, where the regulatory unit has been measured in both cytosol and membrane fractions (344).

The antibody (Steiner RII# 9) was incubated overnight in a range of dilutions on 6µm sections from a rat cerebellum, with equivalent concentrations of non-immune goat immunoglobulin. An FITC rabbit anti-goat serum IgG conjugate (Miles Laboratories, Indiana, U.S.A) was employed at a dilution of 1:30, in place of the usual second antibody. No difference could be found between immunofluorescent staining with

Figure 58 Immunofluorescent localization of the catalytic sub-unit of cyclic AMP-dependent protein kinase in rat cerebellum.

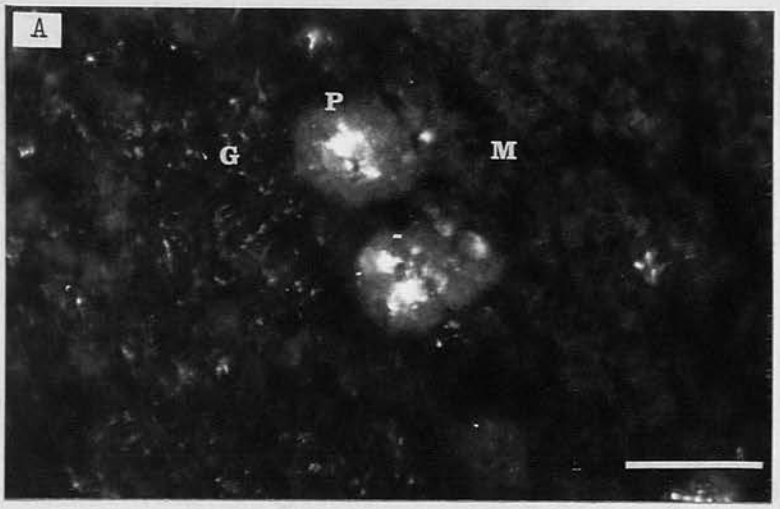
a) Catalytic sub-unit antiserum (Steiner#11).

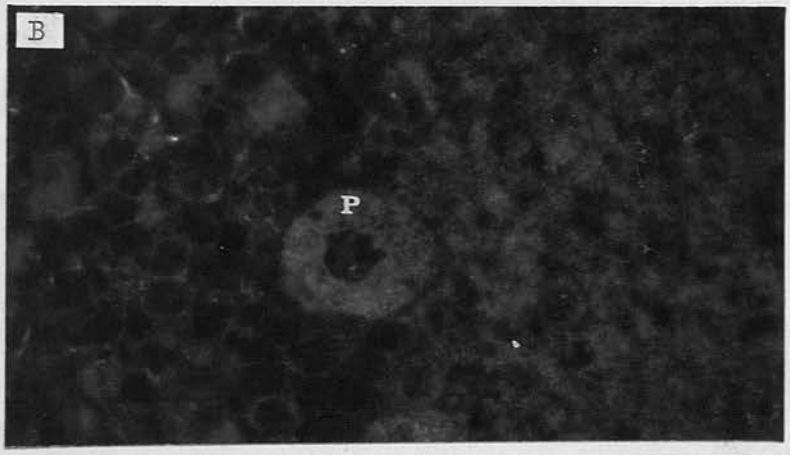
b) Catalytic sub-unit antiserum incubated with excess catalytic sub-unit antigen in liquid-phase, as control.

c) Cerebellar white matter - catalytic sub-unit antiserum.

Calibration bar = 25μm

G - granule layer
M - molecular layer
P - Purkinje cell body





immune and non-immune immunoglobulins, however.

Other RII regulatory unit antibodies will need to be evaluated to determine whether this is a general observation or not, and further immunofluorescence and biochemical studies will be required to elucidate the relation of cyclic AMP to cyclic nucleotide receptor sub-units in the C.N.S., under 'control' and 'stimulated' conditions.

4. EXPERIMENTS TO DETERMINE THE MECHANISM OF BINDING OF CYCLIC GMP IN RAT CEREBELLAR SECTIONS.

4.1. Effect of cyclic GMP and related nucleotides on tissue sections, prior to immunohistochemical processing.

To determine whether 'receptors' for cyclic GMP ' in vitro', could bind exogeneous nucleotide, cyclic GMP at 10mM concentration was added to tissue sections and incubated for 2 hours at room temperature, followed by buffer washing and processing for cyclic GMP immunofluorescence. No change was observed in the intensity and/or distribution of specific staining (see figure 59), suggesting that 'receptors' were not able to bind, and show altered immunofluorescence of cyclic GMP, under these conditions.

Pre-treatment with 10mM concentrations of AMP, ADP, ATP, GMP, GDP and GTP similarly had no effect on the intensity and/or distribution of specific cyclic GMP immunofluorescence.

These observations contrast with those of Sternberger (218), who demonstrated increased immunofluorescent staining for LH-RH on pre-treating rat pituitary tissue sections with the peptide. Pre-treatment with certain analogues removed staining due to displacement of LH-RH from the tissue receptor. Receptors to other molecules have been similarly demonstrated in a number of tissues e.g. prolactin (351) and vasopressin (322).

Figure 59 Effect of pre-treatment of rat cerebellar tissue sections with PBS (control) or 10mM cyclic GMP, prior to immunofluorescent staining.

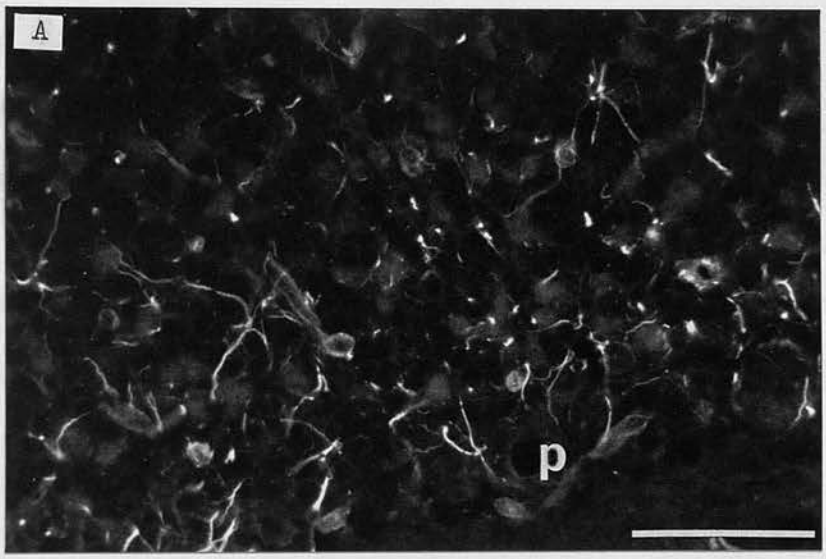
Sections processed with Steiner G17 12/2/74 cyclic GMP antibody.

a) Pre-treatment with PBS (control) for two hours at room temperature.

b) Pre-treatment with 10mM cyclic GMP for two hours at room temperature.

Calibration bar = 50μm

P - Purkinje cell body.



To determine whether tissue sections might bind cyclic GMP but not show changes in immunofluorescence, 6μm rat cerebellar sections on coated glass slides, were incubated with [^{3}H] cyclic GMP or pre-washed overnight in PBS using a magnetic stirrer, to attempt to remove any bound cyclic GMP and expose binding sites, prior to application of the tracer. 0.3 p moles of [ribose-5-^{3}H] cyclic GMP (TRK 499, Radiochemical Centre, Amersham, Buckinghamshire, specific activity: 31Ci/m mole) were applied to tissue sections in a volume of 70μl for 30 minutes or overnight (15 hours), at room temperature. After incubation, a 50μl aliquot of tracer was carefully removed and counted for radioactivity (see page 134). For control, 70μl of tracer were placed on an area of the glass slide without the tissue section, and after the specified incubation period, a 50μl aliquot was removed for counting of radioactivity.

The results shown in table 31, suggest that under the conditions employed, rat cerebellar tissue sections do not show significant uptake of exogeneous cyclic GMP. Two limitations of this method must be realised however: i) conditions of incubation of sections e.g. temperature, may be unsuitable for binding ii) the uptake of tritiated cyclic GMP may be minimal, and undetected using the quantity of tracer employed in the experiment.

Considered together with the 'in vivo' and 'in vitro' data in chapter V, where endogeneous cyclic GMP levels have been increased or decreased without alterations of immunohistochemical cyclic GMP, the 'bound' i.e. immunolocalizable pool of cyclic GMP, does not appear to be influenced by the soluble, or loosely-bound pool.

Table 31 To determine whether individual 6μm rat cerebellar sections show uptake of [^{3}H] cyclic GMP, after pre-incubation under a number of conditions - 0.3 p moles cyclic GMP incubated per tissue section.

Values expressed as counts/4minutes.

	30 minute incubation of frozen section	16 hour incubation of frozen section	16 hour PBS buffer washing of frozen section, followed by 30 minute incubation
50μl [^{3}H] cGMP CONTROL	21,440	21,928 21,280	20,797 21,167
50μl [^{3}H] cGMP AFTER INCUBATION WITH TISSUE SECTION	21,174	22,453	19,670 20,310

4.2. Effect of cyclic nucleotide phosphodiesterase on tissue sections, prior to immunohistochemical processing.

In tissue sections, the constant staining patterns of cyclic nucleotide immunofluorescence with incubation times from minutes to many hours (215), suggests that phosphodiesterase is not active, or is not present at the same sites as the nucleotides. To determine whether tissue cyclic GMP could be degraded by exogeneous phosphodiesterase however, sections of rat cerebellum were incubated with the enzyme prior to tissue processing for immunofluorescence. This technique would also function as a specificity control, since the enzyme will only cleave 3', 5' phosphodiester bonds and should only specifically remove 3',5'

cyclic nucleotide immunofluorescence (See also page 237).

In one experiment, frozen sections were washed in buffer to remove the soluble pool of cyclic GMP, and individually incubated at room temperature for 5 hours, with 0.3 units of phosphodiesterase (Sigma Chemical Co) in a volume of 25μl, or buffer control. After PBS buffer washing (3 x 3 minutes), sections were processed for cyclic GMP immunofluorescence using Steiner G17 7/15/74 antibody.

In a second experiment, frozen sections were washed in buffer to remove the soluble pool of cyclic GMP, and individually incubated overnight at room temperature with 0.3 units of phosphodiesterase in a volume of 25μl, or buffer control. After PBS buffer washing (3 x 3 minutes), sections were fixed in 2% freshly depolymerized paraformaldehyde (see appendix 3). Following 3 x 3 minute PBS buffer washes, sections were processed for cyclic GMP immunofluorescence using Steiner G17 12/2/74 antibody. In both experiments carried out, a slight reduction was observed in the intensity and distribution of positive staining (see figure 60), although background staining was moderately increased.

It is surprising that the large amount of enzyme employed in these experiments only produced a small effect on immunofluorescence. Similar difficulties have been experienced (331) with this approach to the use of the enzyme as a specificity control for cyclic nucleotide immunofluorescence. It seems likely that the 3', 5' phosphodiester group of the bound cyclic GMP is protected from attack by the enzyme, as a result of its orientation in the tissue section. It should also be noted that cyclic AMP when complexed with cyclic AMP-dependent protein kinase, is resistant to attack by phosphodiesterase (39). It would be of interest to confirm the stability of the tissue-bound nucleotide to phosphodiesterase, by assaying cyclic GMP on tissue sections after pre-treatment with enzyme.

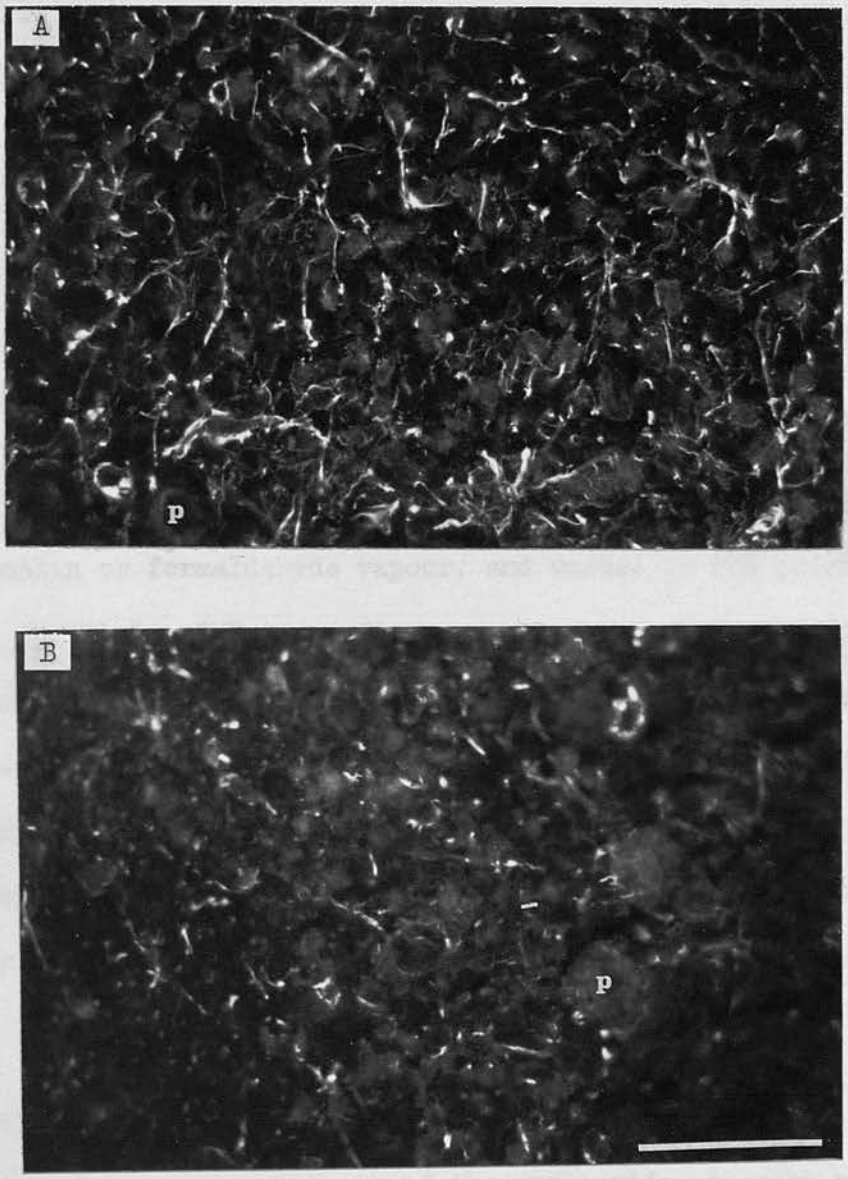
Figure 60 Effect of pre-treatment of rat cerebellar sections with cyclic nucleotide phosphodiesterase prior to immunofluorescence.

a) Section incubated with PBS for 5 hours, followed by processing with Steiner G17 7/15/74 cyclic GMP antibody.

b) Section incubated with 0.3 units of phosphodiesterase for 5 hours, followed by processing with Steiner G17 7/15/74 antibody.

Calibration bar = 50μm

P - Purkinje cell body



4.3. Effect of fixation of tissue sections on immunohistochemistry.

The effect of fixation on cyclic GMP immunohistochemistry has been examined, primarily to determine whether the soluble pool of cyclic GMP could be prevented from being lost from tissue sections. 2% freshly depolymerized paraformaldehyde (see appendix 3) as employed by Wedner et al (235), before or after the cyclic GMP antibody, produced similar results to unfixed rat cerebellar sections, under conditions where cyclic GMP levels were basal or stimulated (see figure 49). This fixative has been employed when section pre-treatment (e.g. with phosphodiesterase) has necessitated incubation with solutions in excess of 15 hours, or when soft tissues (e.g. from 10 day old rats) have been processed for immunofluorescence. Higher concentrations of the fixative have been reported to prevent localization of cyclic GMP in other tissues (215).

Frozen sections of rat cerebellum treated with 'ice-cold' 80% ethanol-water, formalin or formaldehyde vapour, and washed in PBS prior to processing for cyclic GMP immunofluorescence, have abolished positive staining (see figure 61). Similarly, sections taken from the cerebellum of a rat perfused with cold 4% paraformaldehyde in 0.1 M phosphate buffer + 5% sucrose, have shown no specific staining for cyclic GMP. Similar results have been reported using gluteraldehyde, acetone or alcohol for cyclic nucleotide immunofluorescence in other tissues (215).

These fixation techniques may modify a protein associated with cyclic GMP in the tissue, causing release of the nucleotide, thereby preventing positive staining. It may be noted that 80% ethanol-water has been used to extract cyclic GMP from buffer-washed tissue sections for assay.

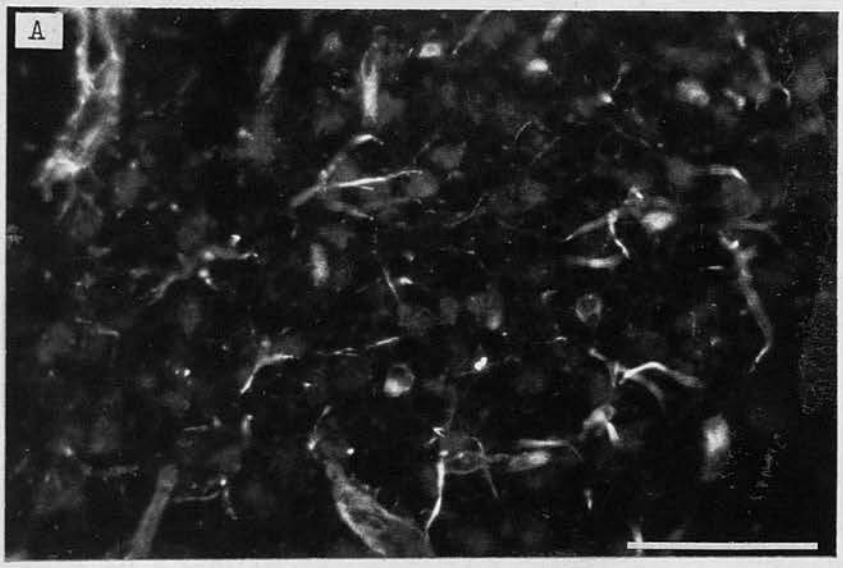
Figure 61 Effect of ethanol fixation of rat cerebellar sections on cyclic GMP immunofluorescence.

a) Unfixed frozen tissue section processed with Steiner G17 12/2/74 antibody.

b) Frozen tissue section fixed with 80% ethanol-water prior to processing with Steiner G17 12/2/74 antibody.

Photographs show cerebellar granule layer.

Calibration bar = 50 µm



Fixatives may modify the primary amino group on the purine moiety of cyclic GMP, preventing binding of specific antibody.

4.4. Effect of acetylation on immunohistochemistry.

Rosenberg et al (331) have shown that gas-phase acetylation of tissue sections, significantly increases the intensity of localization of cyclic AMP in rat liver by immunofluorescence, and that acetylation increases the avidity of cyclic nucleotide antibodies by RIA. This is to be expected, since cyclic nucleotide antibodies are raised against succinyl derivatives of the nucleotides, coupled to proteins. Whilst they have only shown a small effect for cyclic GMP, their experimental technique was carried out to determine whether acetylation affected fibre or capillary cyclic GMP staining components in rat cerebellum.

6μm frozen cerebellar sections were incubated for 5 minutes at room temperature in a Coplin jar containing gauze soaked in triethylamine, followed by 40 seconds in acetic anhydride vapour, using the Coplin jar arrangement. After washing sections for 3 minutes in PBS they were processed, together with controls, using overnight incubation with Steiner G17 12/2/74 antibody. After staining, neither fibre nor capillary component was enhanced, but background staining was slightly increased. Gas-phase acetylation of sections processed with non-immune immunoglobulin, showed a similar increase in non-specific background staining.

4.5. Effect of divalent cations on tissue sections, prior to immunohistochemical processing.

To determine whether divalent cations play an important part in the binding of cyclic GMP to tissue sections, rat cerebellar sections were treated with PBS containing $10^{-3}/10^{-4}$M $CaCl_2$, 10^{-3}M $MgCl_2$ or 10^{-4}M EDTA

for 30 minutes at room temperature, or 37°C (Petri dish containing slide, incubated in an oven at this temperature), prior to processing for immunofluorescence with Steiner G17 12/2/74 antibody. In contrast to the results reported by Steiner et al (215) in liver, no differences were found in positive staining between sections pre-treated with the ions and chelating agents, compared with sections incubated with PBS as control.

4.6. Discussion.

The results of this series of experiments suggests that cyclic GMP is firmly bound in C.N.S. tissue sections, possibly to a protein, which protects the 3', 5' phosphodiester bond from cleavage; the mechanism of binding in C.N.S. sections shows differences compared with binding in liver sections. Since the distribution of the cyclic GMP kinase receptor protein does not parallel that of cyclic GMP, one must postulate a different binding mechanism in the C.N.S.

Complex of cyclic GMP with phosphodiesterase would appear to be unlikely, since the molecules have a different histochemical distribution in the C.N.S. (see reference 248). In regenerating liver, there is a good correlation between cyclic GMP immunofluorescent distribution, and the quantity and activity of particulate guanylate cyclase, but not the soluble form (245). Since the pool of cyclic GMP localized by immunofluorescence is firmly bound to the tissue section, this correlation might suggest that it is synthesized and bound, perhaps in a molecular complex with the cyclase. In the testis, however, increased cyclic GMP immunofluorescence was found to correlate with the soluble guanylate cyclase and not the particulate form (352).

5. GENERAL DISCUSSION

Cyclic GMP and cyclic GMP-dependent protein kinase have been shown in these studies to have a completely contrasting distribution in the C.N.S. by immunohistochemistry, suggesting that the nucleotide is not bound to the kinase holoenzyme in C.N.S. tissue sections. The localization observed with the kinase and catalytic sub-unit of cyclic AMP-dependent protein kinase is particularly interesting, and more extensive studies with more antisera, may be of more use for understanding cyclic nucleotide function in the C.N.S., than for studying the immunohistochemical localization of the individual nucleotides.

The immunohistochemical localization of cyclic GMP kinase, for example, may reflect sites where soluble neuronal cyclic GMP binds transiently, to affect a physiological response. In liver, cyclic GMP and cyclic GMP kinase have been found at the same sites (personal communication Dr Steiner), and perhaps changes in cyclic GMP distribution in tissues will only be observed when there is parallel localization of cyclic GMP and receptor protein; studies on other tissues will confirm if this is a general phenomenom.

A note of warning should be added at this point, however, and that is that analogous to the nucleotides themselves, immunohistochemistry will only localize the tissue-bound pool of the receptor proteins, and this may represent a fraction of the total. Future studies will have to ensure that total protein is localized and, if not, whether fixation can prove to be of use.

Assuming that cyclic GMP is not bound to cyclic GMP kinase in tissue sections, an alternative form of binding that is stable, modified by various fixatives and results in protection of the 3',5' group must be postulated. Since the cyclic nucleotide kinases are the only specific tissue receptor proteins for cylic AMP and cyclic GMP in the C.N.S., a more generalized form of binding may occur, presumably via the charged phosphate groups, or purine nucleus. It must be remembered,however,that the binding has specificity in that: i) cyclic AMP and cyclic GMP show entirely different localization in the C.N.S. ii) species-specificity has been demonstrated for the cyclic GMP fibre-staining component.

The immunofluorescent localization of cyclic GMP to astrocytes, capillaries and epithelial cells of the choroid plexus, has led to the consideration that the nucleotide may be bound to, or associated with, the proteins actin and myosin in C.N.S. tissue sections, since:

i) In the rat C.N.S., smooth muscle actin and myosin have been localized by immunofluorescence to astrocytes, capillaries and blood vessel walls (353, 354). Similar localization has also been found between myosin and GFAP (332).

ii) Actin and myosin-like filaments have been found in rat brain capillaries using electron microscopy (355, 356). Myosin has been found biochemically in a cloned rat glial cell strain from the C.N.S. (357).

iii) In skeletal, cardiac and smooth muscle, cyclic GMP and myosin have beenlocalized by immunofluorescence at the same sites. Ong and Steiner (358) have suggested that cyclic GMP may interact with myosin in some manner, perhaps regulating divalent cation availability, and have a role to play in the contractile process.

iv) In renal cortex, the immunofluorescent localization of cyclic GMP has led to the suggestion that it may be involved in the contractile function of the mesangial cells (240).

v) Actin is localized by immunofluorescence in choroid plexus epithelial cells (359).

An attractive possibility is that the calcium-dependent binding protein, CDR, might be associated with cyclic GMP and myosin. This protein, which is an activator of cyclic nucleotide phosphodiesterase (44), is a sub-unit of myosin light-chain kinase (360); this kinase has recently been partially purified from bovine brain (361). Although CDR has not yet been localized in the C.N.S. by immunofluorescence, studies in culture have shown that it is associated with contractile elements in the cell (362). Perhaps CDR could confer calcium sensitivity on motile phenomena in muscle and non-muscle systems, regulated by myosin, and possibly cyclic GMP.

In attempting to understand the reason for differences in cyclic GMP localization at different tissue sites, an observation from Professor U Groschel-Stewart's laboratory (personal communication) is of particular interest. This group has found that antibodies to smooth muscle myosin from different rabbits, may also show contrasting patterns of immunofluorescent localization of the protein in rat C.N.S. Using one antibody, capillary and blood vessel wall staining was observed, whilst with another, localization was confined to pericytes and astrocytes. Perhaps different forms of myosin at different sites, are associated with cyclic GMP, eliciting stereospecific differences in the determinants

on the bound nucleotide. In this connection it may be noted that myosin exhibits antigenic polymorphism at different sites in cardiac muscle (363).

An association of actin and myosin with cyclic GMP, might suggest a function of the bound nucleotide pool detected by immunofluorescence. Astrocytes in culture, have been shown to change shape, be pulsatile and migratory (364), and contractile responses of glia have been observed in brain slices (365). An actin-myosin contractile system might be involved in these processes, and could also possibly regulate the diameter of capillaries, to modulate blood flow.

Cyclic GMP is localized to fibrous astrocytes in 10 day old rat cerebellum, and may regulate the movement of migrating neurones via an actin-myosin system e.g. the Bergmann glial fibres may aid the migration of cerebellar granule cells during development (367, 368).

It is interesting that a 'glial maturation factor' from adult brain, which 'triggers' astrocytic differentiation in fetal brains in culture, has recently been shown to elevate cyclic GMP levels in the culture (366).

In the choroid plexus, positive immunofluorescent staining was observed for cyclic GMP in the specialised epithelial cells; since actin has also been found at this site by immunofluorescence (359), it may be postulated that bound cyclic GMP functions in a contractile process involved in secretion or osmotic regulation in the choroid plexus, and perhaps also the astrocytes (369). It is of interest that cyclic GMP has recently been detected in isolated rabbit choroid plexus (370).

CHAPTER VII

CONCLUSION

Even though an extensive amount of information is available on cyclic AMP and cyclic GMP in the C.N.S., it is apparent that the role of these molecules in cellular regulation is still not completely understood. The most obvious fact to emerge is that these two structurally related molecules have different roles to play in the C.N.S. Whilst biochemical studies have demonstrated quantitative changes under a plethora of different conditions, the cellular location and sub-cellular distribution of these changes are surprisingly poorly defined in the C.N.S., which contains a variety of cell types. In measuring a change of cyclic GMP levels in the cerebellum for example, it is essential to determine whether the nucleotide is being stimulated in one, or more, of the five neuronal cell types (see reference 346), glial, or even the vascular elements, if we are to determine its function 'in vivo'.

The application of a specific and sensitive immunohistochemical technique enabling localization of cyclic nucleotides in tissue sections, is an attempt to satisfy this vital gap in our knowledge of cyclic nucleotide function.

The studies reported in this thesis have concentrated on the immunofluorescent localization of cyclic GMP in the C.N.S., and have demonstrated two important concepts:

i) Different antibodies differ in their ability to detect cyclic nucleotides in tissue sections, even at different sites and in different tissues, probably as a result of sterochemical differences between bound nucleotides. The binding of an antibody to nucleotide when free in solution, as studied in radioimmunological techniques, is not a suitable 'model' for studying binding to tissue-bound nucleotide.

ii) Immunohistochemistry only detects a tissue-bound pool, which is a fraction of total nucleotide in a C.N.S. tissue section, failing to localize 'stimulated' nucleotide which is probably present in a soluble or loosely-bound neuronal compartment, and therefore lost from the tissue section during processing (the concept of compartmentalization of cyclic nucleotide pools has recently been discussed; see reference 371).

These results clearly limit the value of the immunohistochemical technique for studying cyclic nucleotide function in the C.N.S., and suggest a cautionary approach to studies in other tissues, and also with other antigens. The introductory studies employing antisera to the cyclic nucleotide receptor proteins, suggest that this approach at the cellular and ultra-structural level, might be more lucrative in order to determine where the nucleotides bind and function.

The question remains, however, as to the function of a pool of cyclic GMP detected on tissue sections by immunofluorescence. Whilst functions have been postulated to account for the astrocytic, capillary and choroid plexus epithelium staining in the C.N.S., examination of larger numbers of positive staining antibodies as a result of an intensive immunization programme, must seek to satisfy the immunohistochemical criteria of specificity, and determine whether cyclic nucleotides might be tissue-bound at other loci, hitherto undetected.

Shortly before submission of this thesis, a paper was published by V Chan-Palay and S L Palay entitled:

"Immunocytochemical localization of cyclic GMP: light and electron microscope evidence for involvement of neuroglia"

Proc. Natl. Acad. Sci, U.S.A. (March 1979) 76: 1485-1488

A brief summary of the paper is presented, for comparison with the results reported in this thesis:

Antibodies to cyclic nucleotides were raised by Dr A Guidotti against 2'-O-succinyl cyclic AMP/GMP coupled with keyhole limpet hemocyanin. Indirect immunofluorescence and PAP techniques were employed, on formaldehyde perfused-fixed rat cerebellar tissue.

Positive cyclic GMP staining was found most prominently in the Bergmann glial cell bodies, and their fibres in the molecular layer, staining also being observed in other neuroglia, and some stellate and basket cell somata. Immuno-electron microscopy revealed both membrane and cytoplasmic components which were immuno-reactive.

Employing drugs known to affect biochemical cyclic GMP levels in the cerebellum, altered immunohistochemical staining intensity was found in the neuroglial, stellate and basket cells, and neurones in the deep cerebellar nuclei. Whilst Purkinje cells were unstained with most treatments, superfusion of noradrenaline or glutamate over the cerebellar surface, induced staining of Purkinje cells and also granule cell somata.

Positive cyclic GMP staining was removed by liquid-phase absorption with 1mM cyclic GMP.

A contrasting localization of cyclic AMP was found, distributed in Purkinje cells, granule cells, stellate and basket cells, Bergmann glial cells and their processes, and neurones in deep cerebellar nuclei.

APPENDIX

1. 'COATING'SOLUTION FOR MICROSCOPE SLIDES (372)

1g of gelatin was dissolved in 200ml of distilled water by heating to approximately 60°C, with constant stirring. 0.1g of chrome-alum (chromic potassium sulphate) were then dissolved in the solution. On cooling to room temperature, clean glass slides (76 x 26mm, supplied by Chance Proper Ltd) were immersed in the solution, removed and allowed to dry. Whilst the coating solution was unstable even when stored at 4°C,'coated' slides could be stored permanently at this temperature.

2. PHOSPHATE-BUFFERED SALINE (PBS)

0.15M NaCl, 0.01M phosphate buffer,pH 7.2, was prepared by dissolving the following reagents in 1 litre of distilled water: 8.5g NaCl, 1.34g $Na_2HPO_4.2H_2O$ and 0.39g $NaH_2PO_4.2H_2O$.

3. 2% FRESHLY DEPOLYMERIZED PARAFORMALDEHYDE FIXATIVE

8g of paraformaldehyde were dissolved in 400ml of PBS with constant stirring at 60°C, in a fume cupboard. The resulting solution was allowed to cool, covered,and placed'on ice'; fixative was freshly prepared for each experiment.

4. TOLUIDINE BLUE STAINING OF C.N.S. TISSUE SECTIONS (373)

6µm frozen sections on microscope slides,were placed in a Coplin jar containing approximately 2ml of formalin, to expose the sections to the vapour of the fixative; the lid of the Coplin jar was sealed with glycerol. After 1 minute, the slides were removed and placed over glass rods on the top of a staining trough. Sections were then treated with the following solutions, before being mounted in canada balsam,with clean glass cover slips, for light microscopic observation:

i) 100% ethanol, 10-20 seconds

ii) 50% ethanol, 10-20 seconds

iii) Distilled water, 10-20 seconds

iv) 0.2% toluidine blue, 30 seconds

v) 100% ethanol, 10-20 seconds

vi) Xylene

5. ESTIMATION OF PROTEIN BY THE PROCEDURE OF LOWRY et al (374)

i) For preparation of standards, 25mg of bovine serum albumin (fraction V, Sigma Chemical Co) were dissolved in 10ml of buffer as the stock solution. Duplicate standards were prepared in the range of 15-75μg protein per 300μl solution, by dilution of the stock solution.

ii) 300μl of diluted sample or standards were vortex mixed with 3ml of reagent 1; a 'blank' with 300μl of buffer was also used.

Reagent 1: 50 parts A: 1 part B

where:

A = 2% Na_2CO_3 in 0.1N NaOH

B = 50% (1% $CuSO_4 \cdot 5 H_2O$) and 50% (2% COOK.CH(OH).CH(OH).COONa. $4H_2O$)

iii) After mixing and standing for 15 minutes, 0.3ml of reagent 2 were added to the tubes followed by vortex mixing.

Reagent 2: Folin and Ciocalteu's phenol reagent, diluted 1:1.5 with distilled water.

The tubes were vortex mixed and allowed to stand for 30 minutes.

iv) The 'blank' was corrected to zero optical density in a visible-light spectrophotometer (Guilford Instruments, Middlesex) at 750nm. The optical density of standards and samples were then measured, and a graph was plotted of optical density (ordinate) vs. protein concentration (abscissa). The protein concentration of the samples was calculated from the straight line graph, and after correction for sample dilution, the protein concentration of the undiluted samples was obtained.

6. PREPARATION OF RAT LIVER AND CEREBELLUM ACETONE TISSUE POWDERS (194)

Liver and cerebella were obtained from 4 male adult Wistar rats; the organs were washed clear of blood with cold physiological saline, and then diced with scissors in the same medium. The washed, diced material was then thoroughly homogenized with acetone, and the product was filtered. The deposit on the filter paper was then washed through several times with acetone, before being allowed to dry overnight at room temperature. The dried deposit was then ground in a mortar to obtain a fine grade powder.

For absorption experiments with the powder, it was essential to thoroughly wash the powders several times in PBS prior to incubation with antibody, to remove soluble tissue components. Wet, rather than dried powder must be used in these experiments, to reduce non-specific adsorption of globulin molecules.

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IMMUNOHISTOCHEMICAL LOCALIZATION OF CYCLIC GMP IN RAT CEREBELLUM

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SUMMARY

The technique of cyclic nucleotide fluorescence immunohistochemistry has been applied for the specific localization of cyclic GMP in rat cerebellum. We report immunofluorescence associated with fibres and membranes, contrasting with previously reported cytoplasmic localization of cyclic AMP in different cell populations, using a similar technique.

We have been unable to detect changes in cyclic GMP staining in response to post-mortem changes, harmaline and pentobarbitone administration.

A role of cyclic GMP is suggested in membrane ion transport.

INTRODUCTION

A role for cyclic GMP in cerebellar regulatory systems may be inferred from the high concentration of cyclic GMP[2], the presence of a high affinity binding protein in rat cerebellum[3], and a specific cyclic GMP dependant protein kinase which has been found in bovine cerebellum[4]. The involvement of cyclic GMP with a variety of transmitter systems has been suggested from a number of 'in vivo' and 'in vitro' studies[5,6,7,8]. It is interesting that selective changes of cerebellar cyclic GMP independent of cyclic AMP can be obtained by a number of apparently unrelated agents and conditions (e.g. morphine[9], alcohol[10] and diazepam[11]) possibly suggesting involvement with a basic neuronal mechanism. In this context, 'in vitro' studies have indicated that intracellular calcium ion concentration is a major factor in the regulation of brain cyclic GMP levels[12] and data from a number of tissues suggest that cyclic GMP might participate in the feedback regulation of calcium transport[13].

The localization of cyclic GMP within the cerebellum is unknown, although there are lines of evidence suggesting a cyclic GMP pool associated with the Purkinje cells. A 25-30 fold increase in cerebellar cyclic GMP is

seen as rats develop from 12 days old to maturity correlating with the formation of mossy fibre synapses[14]. Studies on mutant, ataxic mice, which have a selective loss of 90% of cerebellar Purkinje cells, with an otherwise intact cerebellar cortex, similarly have very low levels of cyclic GMP, but normal levels of cyclic AMP[15]. Cerebellar microdissection studies employing radioimmunoassay report a distribution of cyclic GMP consistent with the concept of a Purkinje cell associated pool[16].

In this paper, cyclic GMP has been specifically localized in rat cerebellum using the technique of cyclic nucleotide fluorescence immunohistochemistry[17]. We report widespread localization of immuno-reactive cyclic GMP to fibres and membranes in rat cerebellum, a distribution which contrasts markedly with the previously reported localization of cyclic AMP using the same technique[18,19].

In an attempt to correlate changes in specific staining distribution and/or intensity with biochemical changes of cyclic GMP, we have studied post-mortem change and two pharmacological agents, harmaline and pentobarbitone, which have been shown to produce significant changes of cyclic GMP in cerebellum[14,20,22].

MATERIALS

Isopentane - BDH Chemicals, Poole, England

Paraformaldehyde - BDH Chemicals, Poole, England

Fluorescein isothiocyanate conjugated goat anti-rabbit immunoglobulin (Lot 17), Miles Laboratories, Inc., Miles Research Division, Elkhart, Indiana.

Harmaline - Sigma, London

Pentobarbitone - May and Baker, London

A Zeiss fluorescent microscope was used fitted with BG3 (Schott) primary and 50 (Zeiss) secondary filters, and x10 and x40 Neofluar objectives. Black and white photographs were taken using a 35 mm Olympus OM-1 camera using Ilford FP4 film with a constant exposure time of three minutes.

METHODS

200-250g male Wistar rats were used.

Specific localization of cyclic GMP in cerebellum was detected using an indirect immunofluorescence technique[17]. Highly specific antisera to cyclic GMP were raised in rabbits by repeated injections of 2'O -succinyl cyclic GMP conjugated to keyhole limpet hemocyanin[21]. Immunoglobulin (Ig) fractions of these antisera with high titre were used as the primary immuno-

reagent for cyclic GMP localization.

Rats were sacrificed by decapitation, and cerebella were removed and frozen in isopentane pre-cooled with liquid nitrogen. 4-6μ cryostat sections were then cut at -25°C in a cryostat, and thawed onto albumen coated glass slides. After air-drying a 1:8 dilution of Ig in phosphate-buffered saline (PBS) at pH 7.4 was placed on the unfixed sections and incubated at room temperature in a moist chamber. After 30 minutes, sections were washed for 3 minutes in PBS. Washings were repeated 3 times. Sections were then fixed by immersion in ice-cold 2% freshly depolymerized paraformaldehyde in PBS for 4 minutes. Sections were washed in PBS as before. This fixation procedure unaffected the specific fluorescence but improved tissue morphology. Sites of bound rabbit immunoglobulin were located by incubation with a 1:10 dilution of fluorescein conjugated goat anti-rabbit Ig for 30 minutes, followed by washing as previously described. Slides were then mounted with cover slips using PBS-glycerol (1:1) and sections were viewed using dark field fluorescent microscopy employing episcopic illumination.

Several controls were carried out to establish the specificity of the staining procedure.

No staining was observed when sections were incubated with an equivalent concentration of immunoglobulin from an unimmunized rabbit. If fluorescein labelled anti-rabbit immunoglobulin was reacted with sections without prior exposure to rabbit anti-cyclic GMP, no staining was visualised. Constant immunohistochemical staining patterns were obtained using a number of different antisera to cyclic GMP, although intensity of specific staining relative to background tended to differ. This necessitated the selection of antisera for sharp localization of the cyclic nucleotide in brain tissue. As a final control, antibody adsorption using affinity chromatography resulted in removal of the specific staining pattern[17].

To determine the effect of post-mortem change on cyclic GMP staining, cerebella were removed and frozen at different times after decapitation.

Harmaline and pentobarbitone at 40 mg/kg were given 30 minutes and 5 minutes before sacrifice respectively using intra-peritoneal injections. Immunohistochemical localization was compared with that from control, saline injected rats.

RESULTS

Cerebellar sections treated with antibody to cyclic GMP showed a uniform distribution of bright fluorescent staining, associated with fibres and membranes. In the granular and molecular layers of the cerebellar cortex, scattered cell outlines were stained which did not appear to correspond in distribution to the abundant granule cells or characteristic Purkinje cells

(Fig. 1).

In the molecular layer, parallel fibres and radial fibres show specific staining for cyclic GMP (Fig. 2).

In the white matter of the cerebella, specific staining was uniformly distributed in association with fibres and scattered cell outlines (Fig. 3).

Staining was not observed with control sections, (Fig. 4) although non-specific fluorescence associated with blood vessels and the choroid plexus was occasionally seen.

Effect of post-mortem change, harmaline and pentobarbitone. Sections taken from rat cerebella frozen at times after decapitation from 5 seconds to 5 minutes showed no change in distribution or intensity of specific staining.

Similarly, sections taken from rats injected with harmaline or pentobarbitone failed to show any difference from saline-injected controls, detectable by immunohistochemistry.

DISCUSSION

Immunohistochemistry has revealed differential localization of cyclic nucleotides in a number of mammalian tissues[17]. Cyclic GMP tends to be predominantly located in intimate proximity to the cell membrane, and in association with nuclear material, whilst cyclic AMP is often found in the cytoplasm and in plasma and nuclear membrane areas. We are now able to report a differential localization of cyclic nucleotides in rat cerebellum. Positive staining employing antibody to cyclic AMP has been reported with a cytoplasmic distribution within specific cerebellar neurones - the granule cells, Purkinje cells and some deep cerebellar neurones[18,19]. The localization of cyclic GMP which we now report shows widespread association with fibres and membranes throughout the cerebellum.

It should be considered whether or not the immunoreactive cyclic GMP that we are localizing represents the total content of this cyclic nucleotide in the cerebellum[17]. Only cyclic nucleotide which is tissue bound, presumably via specific receptor proteins, will be localized, since soluble or weakly bound nucleotides will be lost from the frozen tissue in the staining procedure prior to paraformaldehyde fixation. Another consideration is that the cyclic GMP will not be localized if any of its antigenic determinants lay 'hidden' in the tissue.

We have employed immunohistochemistry under conditions which have

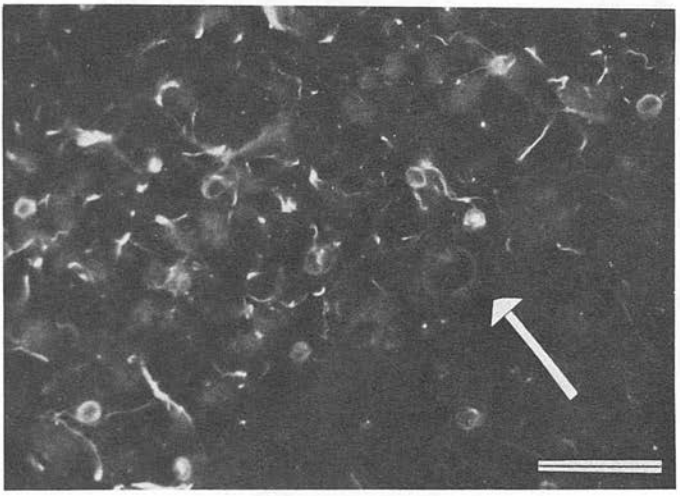

Fig. 1 Immunofluorescent localization of cyclic GMP in the granule cell layer of rat cerebellar cortex. Specific fluorescence is associated with fibres and outlines of scattered cells; the large Purkinje cells are unstained (arrowed). Calibration bar = 25μm.

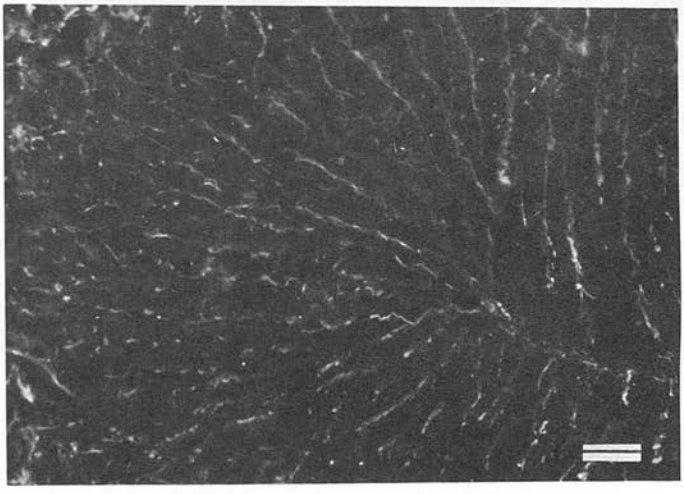

Fig. 2 Immunofluorescent localization of parallel fibres and radial fibres in the molecular layer. Calibration bar = 25μm.

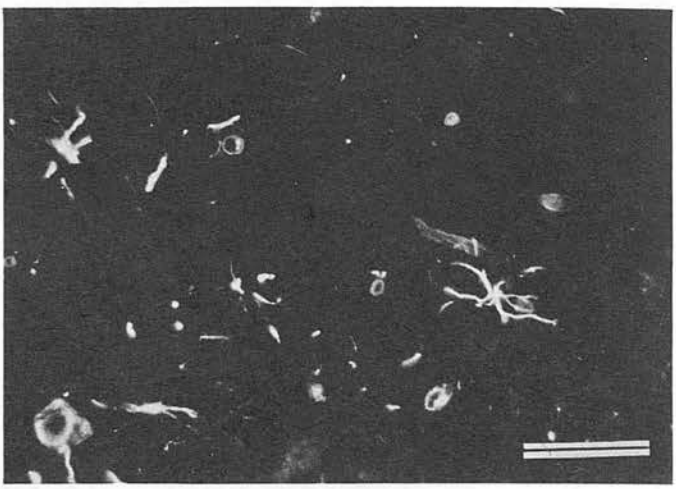

Fig. 3 Specific cyclic GMP localization in cerebellar white matter. Positive staining associated with fibres and scattered cell outlines can be observed. Calibration bar = 25μm.

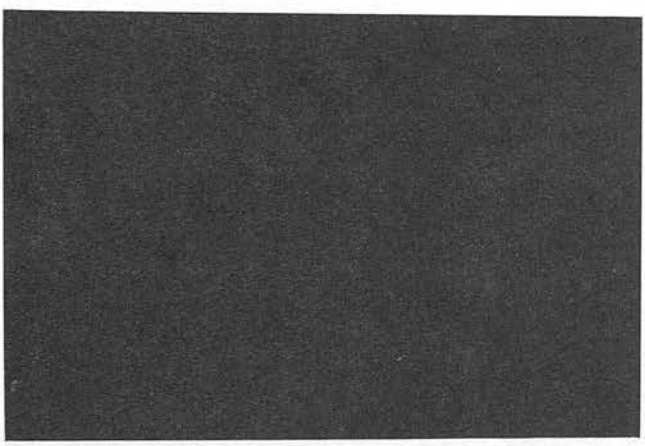

Fig. 4 Control section of cerebellar cortex treated with immunoglobulin from an unimmunized rabbit. Note the absence of specific staining.

previously been shown to signifcantly alter biochemically assayable cyclic GMP[14, 20, 22], and have been unable to detect changes in localization and/or staining intensity. This contrasts with reported modification of cyclic AMP immunofluorescent staining with post-mortem change or pentobarbitone administration. It is unlikely that the technique as applied to brain tissue is unable to detect increased or decreased concentrations of cyclic GMP and possibly more subtle changes in fluorescence intensity within specific cells are occurring which have so far proved undetectable.

Initial studies on the immunohistochemical localization of both cyclic AMP and cyclic GMP in other brain areas have similarly shown contrasting distribution. Cyclic AMP is uniformly distributed cytoplasmically, whilst cyclic GMP is found to be associated with fibres and membranes.

It is interesting that this localization of cyclic GMP would support the hypothesis of an involvement as a regulator of ion transport.

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